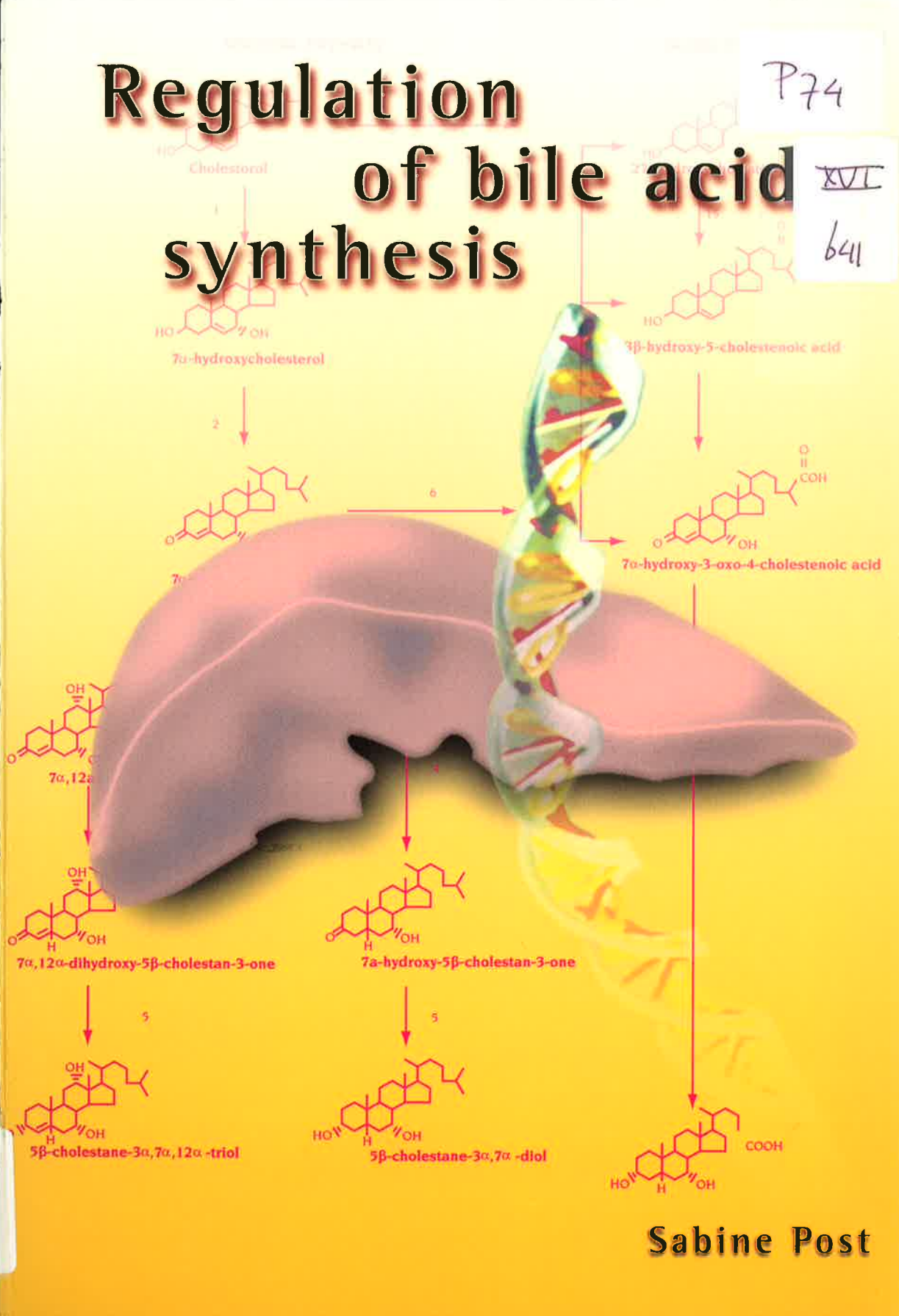
Regulation of bile acid synthesis



Sabine Post

REGULATION OF BILE ACID SYNTHESIS

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THELA · THESIS

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Aan mijn ouders
Voor Martijn

Contents

Outline of this thesis

Conversion of cholesterol into bile acids together with secretion of cholesterol in the bile is quantitatively the major pathway for excretion of cholesterol from the body. Therefore, there is great interest in modulating the bile acid synthetic pathway since this is a way to affect serum cholesterol concentrations beneficially. Our aim was to achieve a better understanding of the regulation of bile acid biosynthesis by different mediators known to have effects on serum cholesterol levels and biliary lipid composition. In this context, we investigated the effect on key-enzymes in the bile acid synthetic pathway i.e. cholesterol 7α-hydroxylase and sterol 27-hydroxylase, that could act as targets for regulation. In addition, the consequences of modulating bile acid synthesis by the different mediators on hepatic lipid metabolism were studied. In the studies described in this thesis *in vitro* as well as *in vivo* model systems were used. Cultured rat hepatocytes, which were previously shown to have comparable features to the hepatocytes *in vivo* with respect to the functional characteristics studied, were used to investigate the direct effect of various modulators on the hepatocytes. In addition, we used different *in vivo* models i.e. rats and transgenic mice to validate *in vitro* findings and to gain more insight into the consequences of regulation for the overall physiology.

Chapter 1 is based on a review article entitled "Regulation of bile acid biosynthesis" which appeared in March 1997 containing the literature reviewed up to September 1996. The current chapter is adapted from this review and recent developments in the field up to April 1999 are included. The main recent developments in the past years added are: 1) The discovery of two different bile acid synthetic pathways involving distinct 7α-hydroxylase enzymes i.e. cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase. 2) The further elucidation of molecular mechanisms by which cholesterol 7α-hydroxylase gene expression is modulated. 3) The role of the recently identified nuclear hormone receptor LXRα in the regulation of cholesterol 7α-hydroxylase gene transcription playing a possible role in cholesterol-mediated induction of cholesterol 7α-hydroxylase. 4) The finding of a linkage between a polymorphism in the cholesterol 7α-hydroxylase gene and high plasma LDL cholesterol concentrations in humans suggesting that genetic differences in the cholesterol 7α-hydroxylase gene may contribute to heritable variation in serum cholesterol concentrations.

In chapter 2 we investigated the direct effect of various lipoproteins which were different in lipid and apolipoprotein content on bile acid synthesis in cultured hepatocytes. When this study was initiated, various investigators reported increases in bile acid synthesis due to cholesterol, however, whether dietary cholesterol exerts its effect directly i.e. via lipoproteins on the hepatocyte or indirectly via malabsorption of bile acids in the intestine, resulting in a reduced potential for bile acid-induced feedback, remained to be clarified. In addition, the effect of lipoprotein cholesterol on sterol 27-hydroxylase expression and the role of different lipoproteins in regulating both enzymes were not well established. So, this chapter deals with the elucidation of the above-mentioned questions.

In chapter 3 the role of cholesterol in the regulation of bile acid synthesis was further investigated by using a novel ACAT inhibitor avasimibe. ACAT is the major enzyme in the esterification of cholesterol, a process which plays an important role in different tissues. ACAT is necessary for proper cholesterol absorption in the intestine and is involved in the accumulation of cholesterol in macrophages in the arterial wall. In the liver ACAT is implicated in the storage of cholesteryl esters and the assembly and secretion of very low-density lipoproteins. Recently, a novel class of ACAT inhibitors with improved bioavailability was developed, able to inhibit both intestinal and hepatic ACAT. Inhibition of ACAT in the liver is thought to enhance the pool of free cholesterol being both a substrate or regulator of bile acid synthesis. Therefore, we studied the direct effect of avasimibe on bile acid synthesis in cultured rat hepatocytes. To gain more insight into the overall effect of avasimibe on lipid metabolism and more specifically bile acid metabolism, and to assess the fate of the free cholesterol in the liver we used in addition to rat hepatocytes the rat as an *in vivo* model.

Cafestol is a coffee diterpene present in unfiltered coffee, which potently increases serum cholesterol levels in humans. The biochemical background of this effect is unknown. Chapters 4 and 5 deal with the unraveling of the cholesterol-raising effect of cafestol. We investigated the potential direct role of cafestol in regulating bile acid synthesis in cultured rat hepatocytes and validated the effects *in vivo* in transgenic mice. One of the strains used is the apolipoprotein E*3-Leiden transgenic mouse. These mice are highly susceptible to diet-induced hyperlipoproteinemia and the lipoprotein profiles from these mice are comparable to those of humans especially in the post-prandial phase. Therefore, we considered these mice to be a good model for investigating the biochemical background of the cholesterol-raising effect of cafestol as observed in humans.

Fibrates are widely used hypolipidemic drugs. It has been established that fibrate treatment causes adverse changes in the biliary lipid composition. The drugs decrease the excretion of bile acids leading to supersaturation of gall bladder bile and consequently to an increased incidence of cholesterol gallstones in patients undergoing long-term therapy. In order to gain insight into the fibrate-induced suppression of bile acid synthesis as observed in humans, we studied the direct effect in cultured rat hepatocytes and further validated the effect *in vivo* in rats and mice (Chapter 6). Fibrates act by activation of specific nuclear receptors, termed PPARs. The direct role of PPARα in this process was investigated in the recently developed PPARα-/- mice. Different mutants of the cholesterol 7α-hydroxylase promoter were of great help in elucidating the PPARα-responsive site in the promoter region of cholesterol 7α-hydroxylase gene.

Female sex hormones are known to inhibit bile flow concomitantly with an excess biliary cholesterol output in humans leading to intrahepatic cholestasis and to an increase in the incidence of cholesterol gallstones. These disturbances could be related to an alteration in bile acid synthesis. In chapter 7 we wanted to assess the effect of the synthetic estrogen 17α-ethinylestradiol on bile acid synthesis and the quantitative contribution of the major pathways in this process in rats. The enzyme activity and mRNA of enzymes involved in bile acid synthesis were measured in livers of control or 17α-ethinylestradiol treated-rats and compared directly to the end result i.e. biliary lipid excretion and composition.

Chapter 1

General Introduction

Adapted from Curr. Pharmaceut. Design 1997;3:59-84

Contents

ABSTRACT

An elevated concentration of low density lipoprotein (LDL) cholesterol is an independent risk factor for cardiovascular disease. The only quantitatively significant way by which cholesterol is removed from the body is via the bile, either directly or after conversion to bile acids. Therapeutic modalities which increase bile acid biosynthesis, e.g. bile acid-binding resins, have been shown to reduce plasma LDL levels and the risk of coronary heart disease. Their effects, however, are limited and patients' acceptibility is poor. This warrants the search for more potent drugs and new ways to enhance bile acid synthesis. In this review the various pathways along which bile acids are formed will be discussed with emphasis on the regulation of cholesterol 7α-hydroxylase, the major rate-limiting step in bile acid formation, as a target to increase bile acid synthesis. In the past 5 years our understanding of the transcriptional mechanism that regulates the expression of this gene has rapidly expanded. In view of the increasing evidence for the substantial contribution of the alternative or 27-hydroxylase route to bile acid synthesis, the current knowledge on sterol 27-hydroxylase, the first enzyme in this pathway and a potentially new target, is reviewed. Several companies are involved in developing new bile acid sequestrants and other inhibitors of intestinal bile acid absorption to remove cholesterol from the body. This review article presents recent developments in this field as appeared in the patent literature during past the years.

1. INTRODUCTION

1.1 Role of bile acid formation in regulation of LDL cholesterol and development of atherosclerosis

An elevated concentration of low density lipoprotein (LDL) in blood is an important risk factor for the development and progression of atherosclerotic cardiovascular disease (1-3). The liver plays an important role in the regulation of plasma LDL levels and different metabolic pathways are integrated in the liver to maintain cholesterol homeostasis in the body (Figure 1). Cholesterol can be synthesized in the liver from acetate, in response to changes in hepatic cholesterol levels, and the major rate-limiting enzyme in this process is 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. Cholesterol, either from exogenous or endogenous origin, can be

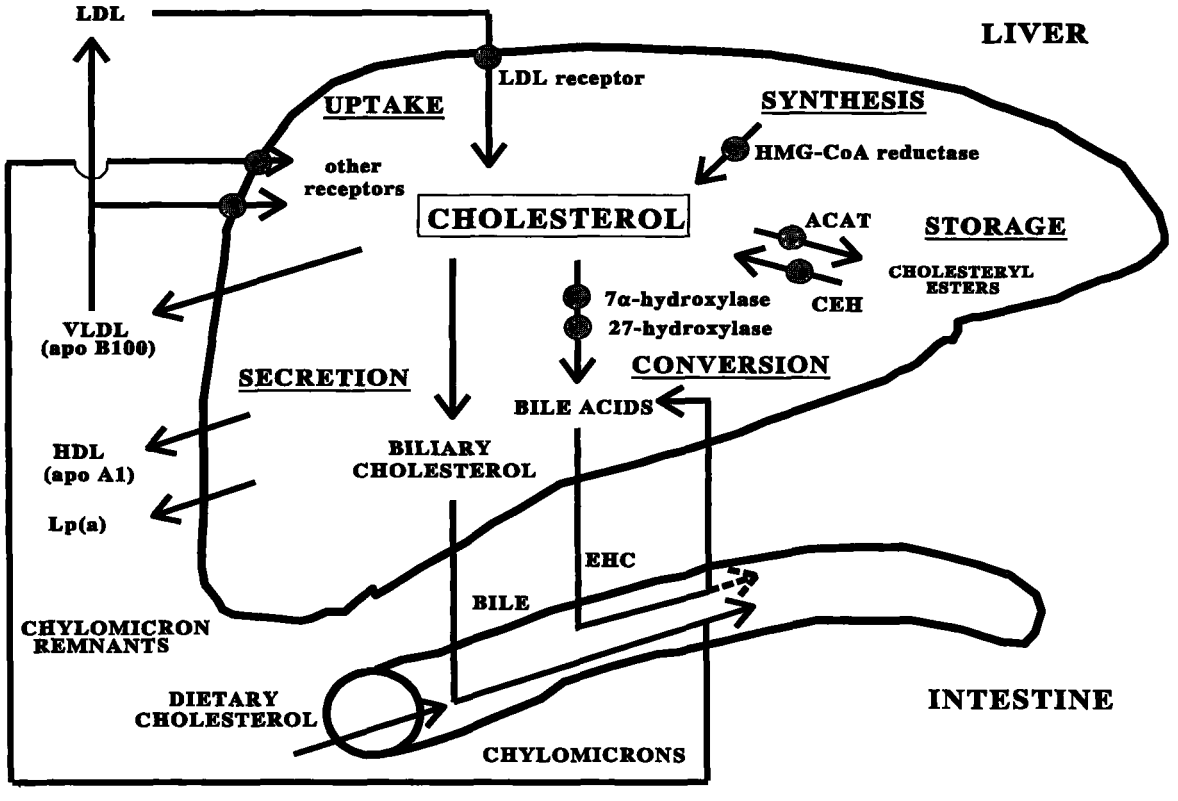


Fig. 1 Schematic representation of cholesterol metabolism in the liver.
ACAT, acyl-CoA: cholesterol acyltransferase; CEH, cholesterylester hydrolase; 7α-hydroxylase, cholesterol 7α-hydroxylase; 27-hydroxylase; sterol 27-hydroxylase; EHC, enterohepatic circulation.

directed to the liver by receptor-mediated uptake of lipoproteins, of which LDL is quantitatively the most important cholesterol carrier in humans. The rate of the LDL receptor-mediated uptake of LDL depends on the balance between influx and efflux of cholesterol in the liver. An excess of cholesterol can be stored in the liver after conversion to cholesteryl esters by acyl-CoA:cholesterol acyltransferase (ACAT). On the other hand, the output pathways responsible for elimination of cholesterol from the liver involve both secretion of cholesterol by lipoproteins, as well as excretion of free cholesterol or cholesterol-derived bile acids into bile.

Biliary bile acid and cholesterol excretion is the only significant way for elimination of cholesterol from the mammalian body (4). Following synthesis, bile acids are actively secreted from the parenchymal cells in the common bile duct (5,6). In man, and monkey, and also pig, mice and hamster, the majority of bile acids is then stored in the gallbladder, to await release upon hormonal stimulation in the duodenum. In contrast, rats do not posses a gallbladder. After excretion in the intestine bile acids play an important role in the solubilization of fats, cholesterol, and other lipophilic compounds such as vitamins A, D, E and K, and drugs enhancing their uptake by enterocytes. Approximately 95% of the bile acids are reabsorbed in the terminal ileum and return to the liver, where they are actively taken up. This cycling of bile acids between liver, gallbladder and intestine is called the enterohepatic circulation (EHC) (7). Biliary cholesterol is less efficiently taken up in the intestine, approximately 50% is absorbed. The total mass of cholesterol which is lost from the human body is estimated to be 1000 mg per day, i.e. 600 mg cholesterol and 400 mg as bile acids (4,7,8). The excretion of cholesterol into bile is coupled to the secretion of bile acids. Consequently, remedies which accelerate bile acid synthesis will also lead to enhanced removal of cholesterol from the liver, which is compensated for by an increased hepatic LDL receptor activity and a decrease in plasma LDL levels in man.

Different studies indicate the importance of bile acid synthetic capacity in cholesterol homeostasis, i.e. regulation of plasma LDL concentration, and in prevention of atherosclerosis. Enhancement of bile acid synthesis in man can be accomplished, by interruption of the EHC of bile acids, either by ileal bypass surgery, as was practized as an experimental therapy in former days, or by administration of the resins cholestyramine or colestipol, binding bile acids in the intestine. Such therapy successfully lowers plasma levels of LDL in man, whereas high density lipoprotein (HDL) levels remain unchanged or slightly increase (9-15). A large clinical intervention study, the Lipid Research Clinics Coronary Primary Prevention Trial with

cholestyramine has shown that the latter treatment indeed decreases the incidence of coronary heart disease (CHD) mortality and non-fatal myocardial infarction (11,12). On the other hand, a low bile acid synthetic capacity was found to be an independent risk factor for the incidence of CHD, and subnormal levels of bile acid synthesis were correlated to the progression of atherosclerosis and coronary mortality in patients heterozygous for Familial Hypercholesterolemia (FH) (16). Additionally, an increase in plasma levels of LDL cholesterol, occuring in parallel with aging in man, has been reported to be inversely correlated with a decrease in bile acid synthetic capacity (17).

Animal studies showed that genetic factors may influence the responsiveness to dietary cholesterol, as is evident from changes in plasma cholesterol concentration, and that this is closely related to the bile acid synthetic capacity. For example, certain strains of mice, which are hypo-responsive to dietary fat and cholesterol by developing only mildly elevated plasma cholesterol levels, had a higher bile acid synthesis as compared to hyper-responders (18). Infection of hamsters (19) or LDL-receptor knock-out mice (20) with a recombinant adenovirus encoding cholesterol 7α-hydroxylase, the major rate-limiting enzyme in the bile acid synthetic pathway, reduces plasma total and LDL cholesterol. Furthermore, non-viral-mediated delivery of a synthetic transgene encoding cholesterol 7α-hydroxylase to hepatocytes of intact hypercholesterolemic mice resulted in a reduction of the plasma cholesterol content (21). In pigs, polymorfisms of cholesterol 7α-hydroxylase were associated with plasma cholesterol levels (22). Recently, Wang *et al.* (23) found a linkage between a polymorfism in the cholesterol 7α-hydroxylase gene and high plasma LDL cholesterol concentrations in humans suggesting that genetic differences in the cholesterol 7α-hydroxylase gene may contribute to heritable variation in plasma cholesterol concentrations.

The above data emphasize the role of bile acid synthesis in the regulation of plasma cholesterol and LDL levels. However, therapeutic modulation of bile acid synthesis with the objective to treat hypercholesterolemia is still in its infancy. The magnitude of the effect of bile acid sequestrants on plasma LDL concentration is limited, usually in the range of 10-20% reduction (9-15), and the drug has a poor patient acceptability (10-12). Side effects may be various gastrointestinal complaints as obstipation, flatulence, epigastric fullness and nausea. This provides a rationale for continuing the search for possibilities to manipulate bile acid synthesis with the ultimate objective to reduce atherosclerosis and possibly gallstone formation. The

latter disease often occurs in humans with supersaturated bile, i.e. bile containing a relatively large amount of cholesterol. Increased bile acid synthesis will theoretically counteract this process. Research into the mechanism involved in regulation of bile acid synthesis may reveal new strategies for development of more efficacious regulators of cholesterol metabolism.

In view of the importance of elimination of cholesterol from the body in diseases as hyperlipidemia and atherosclerosis, and gallstone disease, it is not surprising that several important reviews have been published on biosynthesis, metabolism and pathophysiology of bile acids over the years. We would like to refer to Björkhem 1985 (24), Fears and Sabine 1986 (25), Vlahcevic *et al.* 1992 (26), Russell and Setchell 1992 (27), Björkhem 1992 (28), Hofmann 1994 (29), Carey and Duane 1994 (7), and Okuda 1994 (30). In addition, the role of the liver in maintaining whole body sterol balance and in the regulation of plasma LDL concentration has been reviewed in detail by Turley and Dietschy 1988 (4) and Dietschy *et al.* 1993 (8). The present review will update our understanding of the regulation of bile acid biosynthesis by different mediators, with emphasis on the key enzyme in the bile acid synthetic pathway, cholesterol 7α-hydroxylase, and on sterol 27-hydroxylase, as a potentially new target to influence bile acid synthesis. New developments in drug design to influence these processes will also be discussed. Mechanisms involved in hepatic uptake of bile acids, intracellular bile acid transport, excretion of bile acids and bile formation, and intestinal absorption of bile acids are beyond the scope of this review and will not be discussed. Excellent reviews on advances in our knowledge of the latter processes have appeared in recent years. We would like to refer, therefore, to these references (5-7,29,31-33 among others).

2. PATHWAYS IN BILE ACID SYNTHESIS

The various steps in bile acid synthesis are shortly memorized and updated here. Citations to original papers are curtailed in this item because of space limitations. The enzymatic reactions are extensively described in the above-mentioned reviews (24-28).

The conversion of cholesterol into bile acids involves at least 17 different enzymes, causing a cascade of reactions occuring in the microsomes, cytosol, mitochondria, and peroxisomes. The major primary bile acids formed in humans and e.g. hamsters are cholic and chenodeoxycholic acid, but there are many species-

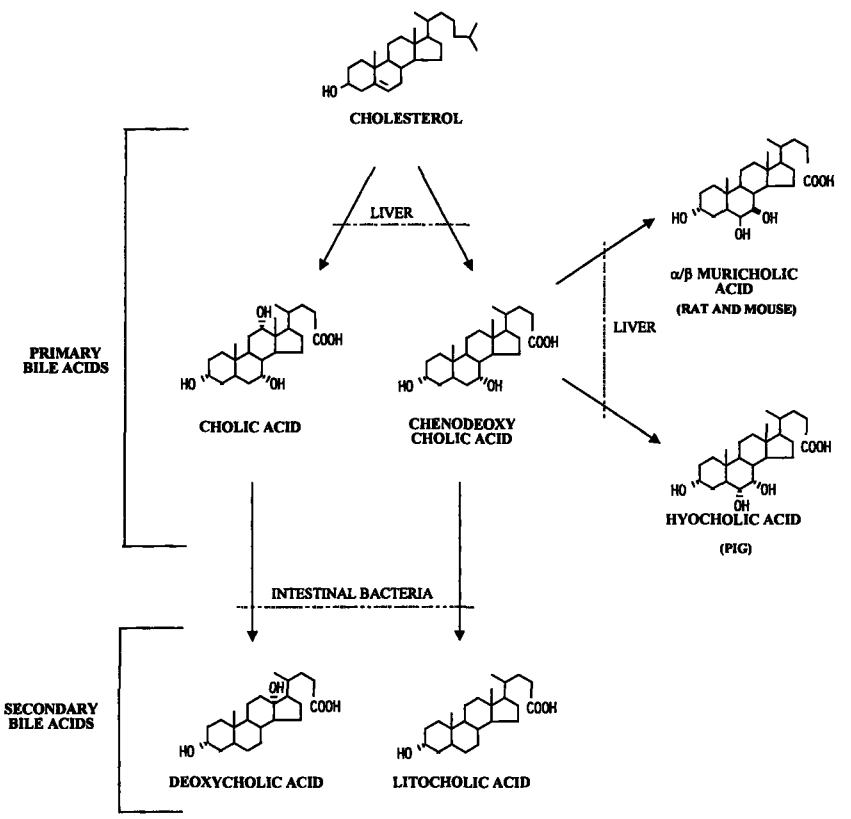


Fig. 2 Major primary and secondary bile acids in humans and selected other species, and sites of synthesis and metabolism.

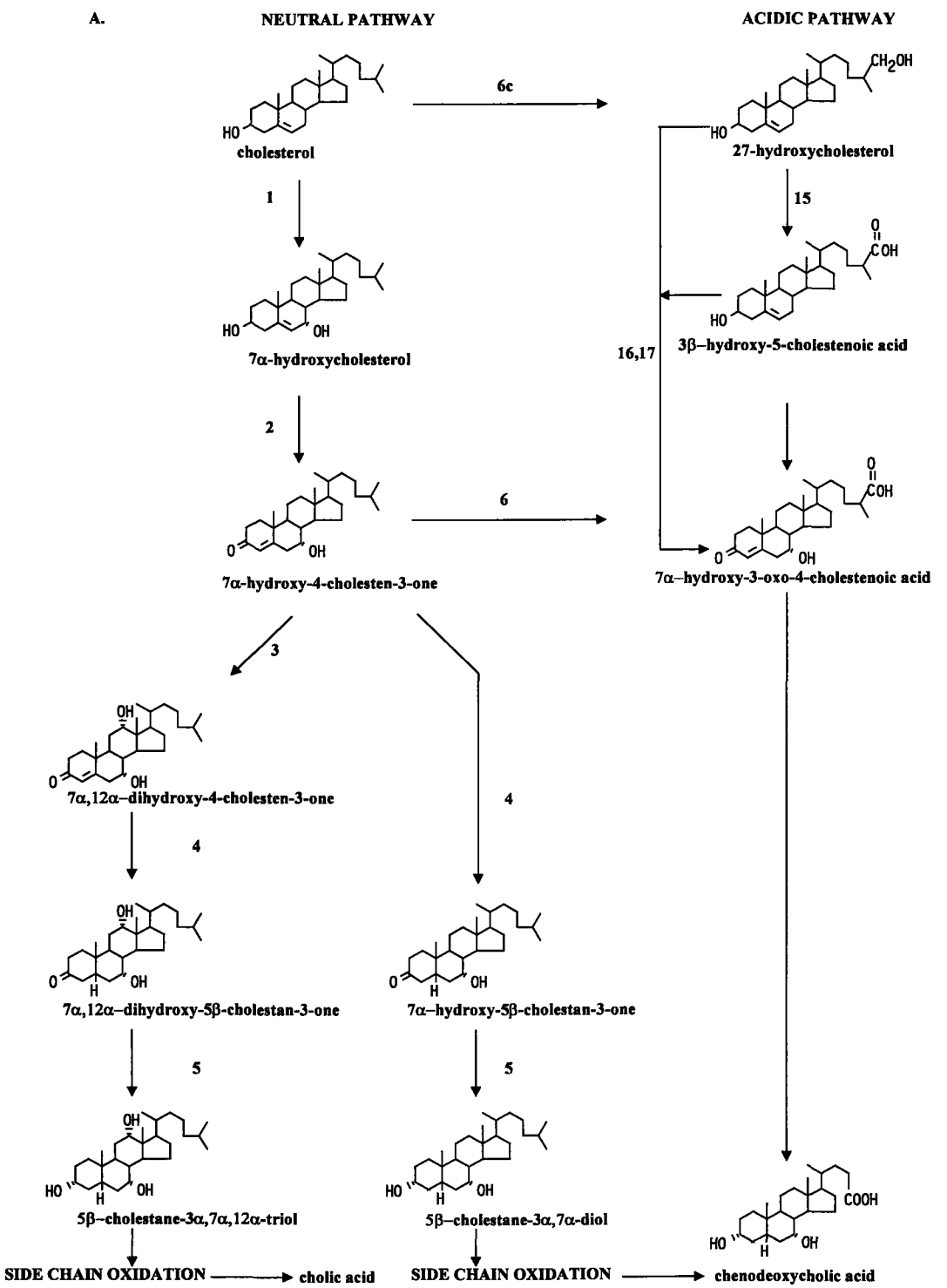
related differences (29). In rats part of chenodeoxycholic acid is converted in α- and β-muricholic acid, while they are converted allmost entirely to the latter in mice. Primary bile acids can be biotransformed in the intestinal tract by bacterial flora forming secondary bile acids, being deoxycholic acid and lithocholic acid from cholic acid and chenodeoxycholic acid, respectively (Figure 2).

There are two different pathways in bile acid biosynthesis. The primary route, named the neutral or 7α-hydroxylase route, is initiated by the rate-limiting enzyme cholesterol 7α-hydroxylase, whereas the alternative or acidic pathway is initiated by the enzyme sterol 27-hydroxylase (Figure 3). The neutral pathway starts with modification of the steroid nucleus, while the acidic pathway is initiated with side chain oxidation and shortening thereof.

2.1 The neutral pathway

The neutral pathway in bile acid synthesis starts with the 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase, which is localized in the smooth endoplasmic reticulum (reaction 1 in Figure 3A). The enzyme is a member of the cytochrome P-450 superfamily and a mixed-function monooxygenase. It utilizes NADPH as an electrondonor and a protein cofactor, cytochrome P-450 reductase for transfer of electrons to the cytochrome P-450 entity, and is considered to be the main point of regulation. Serum concentration of 7α-hydroxycholesterol is used as an indicator of bile acid synthesis in humans during disease and intervention (for recent papers see 34-37). The second step in this route is catalyzed by the microsomal 3ß-hydroxy-Δ^5-C_{27} steroid oxidoreductase (38) (reaction 2). In rats this enzyme is next to cholesterol 7α-hydroxylase important in regulation of the serum 7α-hydroxycholesterol levels. For example, sex differences in serum 7α-hydroxycholesterol in rat were reflected by activity of both these enzymes (39) This enzyme is recently shown to be localized not only in the liver but also in extrahepatic tissues i.e. lung and ovary (40). The product of this reaction, 7α-hydroxy-4-cholesten-3-one, is a branch-point biosynthetic intermediate that can be either 12α-hydroxylated (reaction 3) or converted to 7α-hydroxy-5ß-cholestan-3-one (reaction 4) or to 7α-hydroxy-3-oxo-4-cholestenoic acid (41,42). The plasma levels of 7α-hydroxy-4-cholesten-3-one and of the latter intermediate can also be used as an index for monitoring cholesterol 7α-hydroxylase activity *in vivo* (e.g.41-45). The transformation by the microsomal 12α-hydroxylase leads finally to the formation of cholic acid, whereas the conversion by the soluble enzyme Δ^4-3-oxosteroid-5ß-reductase to 7α-hydroxy-5ß-cholestan-3-one (reaction 4) further follows the pathway which finally results in the formation of chenodeoxycholic acid. The product after the 12α-hydroxylation can also be reduced by Δ^4-3-oxosteroid-5ß-reductase to 7α,12α-dihydroxy-4-cholestan-3-one (reaction 4). Before side-chain degradation both 7α-12α-dihydroxy-5ß-cholestan-3-one and 7α-hydroxy-5ß-cholestan-3-one are subsequently reduced by the NADPH-dependent soluble enzyme 3α-hydroxysteroid oxidoreductase (reaction 5). The final steps in the neutral route involve side-chain degradation, a process taking place mainly in the mitochondria and peroxisomes, and are shared by the latter two intermediates (reactions 6-14).

The first step in the side chain degradation is catalyzed by the mitochondrial cytochrome P-450 sterol 27-hydroxylase (reaction 6a, in Figure 3B). This enzyme was used to be designated as 26-hydroxylase, but it is now more correctly termed


A.
NEUTRAL PATHWAY
ACIDIC PATHWAY
cholesterol
6c
27-hydroxycholesterol
1
15
7α-hydroxycholesterol
3β–hydroxy-5-cholestenoic acid
16,17
2
6
7α-hydroxy-4-cholesten-3-one
7α–hydroxy-3-oxo-4-cholestenoic acid
3
7α,12α–dihydroxy-4-cholesten-3-one
4
4
7α,12α–dihydroxy-5β-cholestan-3-one
7α–hydroxy-5β-cholestan-3-one
5
5
5β–cholestane-3α,7α,12α-triol
5β–cholestane-3α,7α-diol
SIDE CHAIN OXIDATION
cholic acid
SIDE CHAIN OXIDATION
chenodeoxycholic acid

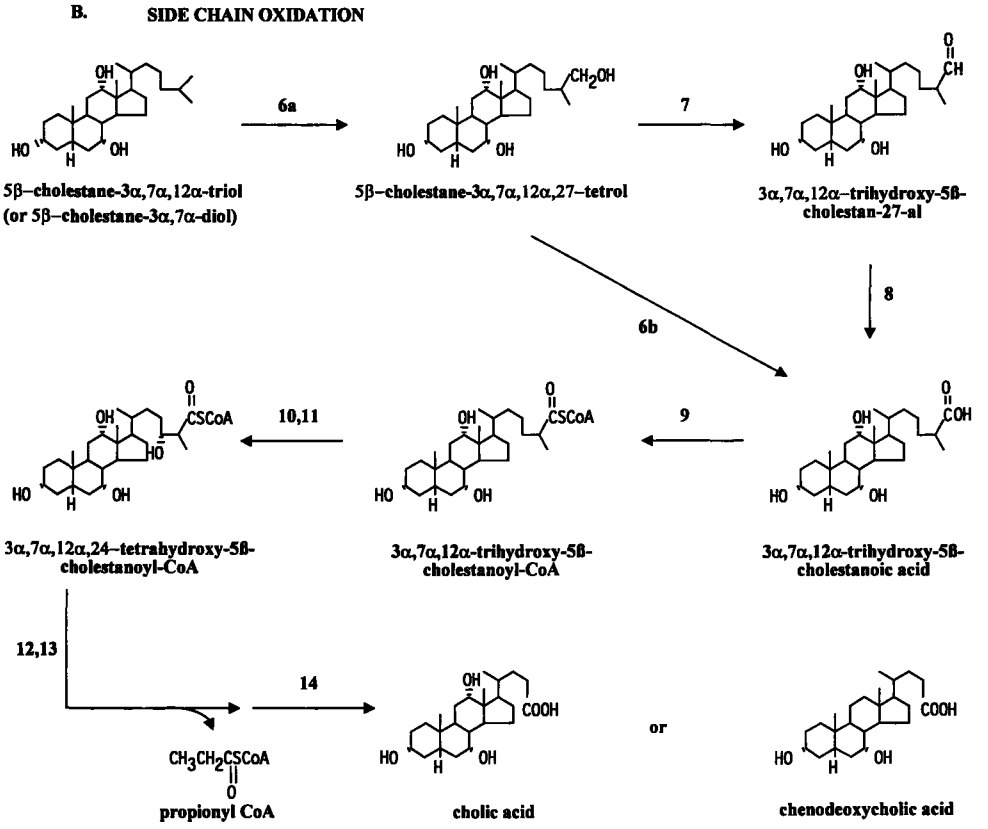


Fig. 3 Pathways to formation of bile acids.

A. Neutral and acidic pathway. B. Side chain oxidation of products generated via the neutral pathway. Conversions depicted can show promiscuity with respect to substrates and may feature in more synthetic routes. Adapted from Russell and Setchell (27)

sterol 27-hydroxylase (24,28), since the enzyme selectively introduces a hydroxyl group at C-27 of 5ß-cholestane-3α,7α,12α-triol and 5ß-cholestane-3α,7α-diol. It does so in the presence of molecular oxygen, NADPH, and two protein factors, ferredoxin and ferredoxin reductase (46). The enzyme is promiscuous as it has affinity for and can oxidize different substrates, including cholesterol and vitamin D_3 (28,30,46-48). The presence of normal levels of vitamin D metabolites in sterol 27-hydroxylase knock-out mice (49) and the very low activity of purified, bacterially expressed human sterol 27-hydroxylase towards vitamin D_3 (50) suggest that sterol 27-hydroxylase is less important in this process. Hence, the enzyme may also be

involved in another pathway in bile acid synthesis, as described below. Sterol 27-hydroxylase will not only catalyze formation of bile alcohols, but it may also act to form the carboxylic acid derivative 3α,7α,12α-trihydroxy-5β-cholestanoic acid (reaction 6b) (46,47). Alternatively, formation of the latter intermediate may result from the action of cytosolic NAD-dependent alcohol dehydrogenase (reaction 7) and aldehyde dehydrogenase (reaction 8). The resulting acid can in turn be converted to thio-esterified 5β-cholestanoic acid in the microsomes (reaction 9).

The terminal steps of the side chain oxidation take place mainly in the peroxisome, following a pathway which is similar to the β-oxidation of fatty acids in this organelle (51). These reactions involve the introduction of a double band between C24-C25 of the CoA ester of 5ß-cholestanoic acid by an oxidase, followed by a dehydrogenase/hydratase to yield a 24-hydroxylated species, 3α,7α,12α,24-tetrahydroxy-5ß-cholestanoyl-CoA (reactions 10 and 11). The formation of this product proceeds via a Δ^{24} intermediate, followed by conversion to a 24-oxo intermediate by a bifunctional dehydrogenase/ hydratase. Subsequent thiolytic cleavage results in the loss of a propionyl-CoA and the formation of a C24 bile acid-CoA product (reactions 12 and 13). Prior to secretion from the hepatocyte, and after formation of CoA-conjugated bile acids by a microsomal CoA synthetase, cholic acid and chenodeoxycholic acid are substrates for the enzyme bile acid-CoA:amino acid *N*-acyltransferase that utilizes either glycine or taurine to form conjugated bile acids. Approximately 98% of the bile acids are excreted in the conjugated form, lowering their pK and solubility at physiological intestinal pH (7).

Thus, within this route, different cellular compartments are involved in the formation of bile acids. How this intracellular trafficking of bile acid intermediates may be envisioned, is at present largely unknown, although the soluble 3α-hydroxysteroid dehydrogenase has been assigned such a function (29).

2.2 The acidic pathway

Based on animal experiments and observations in human serum, in which conversions and bile acid intermediates occured, which could not be explained by the mere existence of the neutral route, an alternative pathway has been proposed. In this case, side-chain degradation precedes biotransformation of the steroid nucleus, yielding acidic C24-intermediates (28,42). This pathway has, therefore, been termed the acidic pathway, in contrast to the classical or neutral pathway, initiated by cholesterol 7α-hydroxylase (Figure 3A).

The catalytic activity of sterol 27-hydroxylase toward cholesterol has been found to be lower as compared to other C27-sterol substrates (24,28,30,47). However, more recent data indicate that substantial amounts of cholesterol, the most abundant substrate in the liver cell can be converted into 27-hydroxycholesterol (reaction 6c) (28,42). The intermediate 27-hydroxycholesterol is converted to 3ß-hydroxy-5-cholestenoic acid in rat liver peroxisomes (reaction 15) (52). This product was also formed in a reconstituted system with the purified *E-coli* expressed human sterol 27-hydroxylase giving first an aldehyde, which is further oxidised to 3ß-hydroxy-5-cholestenoic acid (53). In addition, the 27-hydroxycholesterol can also be converted to 7α-hydroxy-intermediates by liver microsomes and mitochondria from different species (54-61). This 7α-hydroxylase activity has also been found in mouse brain (62), cultured rat ovary cells (63) and human fibroblasts (64). In human microsomes a cytochrome P-450 enzyme, requiring NADPH and cytochrome P-450 reductase catalyzes 7α-hydroxylation of 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid, whereas a mitochondrial enzyme is active in 7α-hydroxylation of 3β-hydroxy-5-cholestenoic acid (54,57,59). Both enzymatic conversions lead ultimately via a 3β-hydroxy-Δ^5-C_{27} steroid oxidoreductase (38) to formation of 7α-hydroxy-3-oxo-4-cholestenoic acid (reactions 16,17).

The acidic pathway gives rise to preferential formation of chenodeoxycholic acid both in rat and man (65-68). It appears that the microsomal 12α-hydroxylase is specific with respect to side chain length. Once the side chain degradation has occured, the enzyme can no longer be active on the resulting products, therefore yielding only chenodeoxycholic acid. The rabbit appears to be an exception, since the major part of 27-hydroxycholesterol is converted into cholic acid in this species (69).

The alternative pathway substantially contributes to bile acid synthesis both in humans (42), in cultured human and rat hepatocytes (70,71), and *in vivo* in rat (72). Under certain pathological circumstances the alternative pathway may even be the major route to bile acid synthesis (35,36,42,73). The importance of the 27-hydroxylase route may have been underestimated in the past, since many studies were performed with rats and rabbits, often after stimulation of bile acid synthesis by biliary diversion or sequestrants. Absence of a gallbladder, in addition to an unusually long small intestine in rat, results in a high bile acid synthetic capacity (4,8). As a consequence of these anatomical differences a major portion of the available cholesterol in the rat is probably diverted to the 7α-hydroxylase or neutral

pathway possibly as opposed to the situation in man. Interruption of the enterohepatic circulation further increases the contribution of the 7α-hydroxylase pathway to bile acid synthesis (34,35,42). Rat and rabbit have considerably lower blood concentrations of 27-hydroxycholesterol than humans (28), and lower activities of enzymes involved in 7α-hydroxylation of intermediates of the acidic pathway have been found in rats and rabbits (57), suggesting that the acidic pathway is less important in the latter species. This contention is supported by the fact that cholic acid is the predominant bile acid in bile of rats (74).

3. MAJOR REGULATORY ENZYMES IN BILE ACID SYNTHESIS

3.1 Cholesterol 7α-hydroxylase

The microsomal enzyme cholesterol 7α-hydroxylase is a member of the cytochrome P-450 superfamily (75,76), and the first and rate-limiting enzyme in the neutral pathway in bile acid synthesis. 7α-Hydroxylation of cholesterol is considered to be the main point of regulation in bile acid synthesis. The recent generation of cholesterol 7α-hydroxylase knock-out mice showed that absence of the gene results in postnatal lethality caused by deficiency in fat-soluble vitamins (73,77). The enzyme is present at birth, decreases to undetectable levels by day 6-8 of postnatal life and reappears around day 12 of suckling, reaching peak levels at weaning (78-80). Cholesterol 7α-hydroxylase is highly specific for cholesterol and cholestanol (81). In 1985 the group of Danielsson and Wikvall purified the protein to near homogeneity (82). Later, the enzyme was obtained in sufficient quantities, to allow sequencing of the protein, production of antibodies against it, and cloning the corresponding cDNA and gene from rat and humans (83-93). The cholesterol 7α-hydroxylase enzyme consists of 503 (rat) or 504 (human) amino acids with a predicted molecular weight of 57 kD and is considered to constitute a novel P-450 member (CYP7A), because of a sequence similarity with other cytochrome P-450 enzymes of less than 30%. Cholesterol 7α-hydroxylase consists of a highly hydrophobic amino-terminal membrane-anchoring region, a heme-binding domain, and a sterol-binding site. These regions were shown to be completely identical in human, rat, and hamster (94). Expression of the human cholesterol 7α-hydroxylase in *E. coli* provides a method for obtaining large quantities of the active enzyme to study the structure function relationship (95). The activity of purified cholesterol 7α-

hydroxylase from this expression system can be modulated *in vitro* posttranslationally by phosphorylation and dephosphorylated (96). Cholesterol 7α-hydroxylase is only detected in the liver (87).

Northern blotting revealed the presence of multiple cholesterol 7α-hydroxylase mRNAs with reported lengths of 1.8, 2.1, 3.6, and 4.0 kb in rat, although reports vary, both *in vivo* (87) and in cultured rat hepatocytes (97). Recently, a transcript of approximately 7 kb was also identified in rat liver (77). In human liver a single mRNA of 2.8 kb was detected (89). The variety in mRNA lengths is due to multiple poly-adenylation sites present in the 3'-untranslated region (UTR), generating mRNAs of variable size (88). Furthermore, the 3'-region of the 4 kb mRNA is rich in AU-sequences containing the sequence motif AUUUA repeated in rat 17 times and 5'-AAU-3' or 5'-UAA-3' trinucleotides (98), which have been linked to rapid degradation of mRNA (99,100).

The half-life of cholesterol 7α-hydroxylase activity and mRNA has been estimated to be 4 hours in rat hepatocytes (97), in line with a reported short half-life of 2-3 hours for the enzyme *in vivo* in bile-fistulated rats, treated with inhibitors of protein synthesis and transcription (101,102).

The gene encoding cholesterol 7α-hydroxylase has been cloned from the rat (91,92), human (93,103,104), hamster (94) and mouse (105). The rat, hamster and human gene all span about 11 kilobases, comprising 6 exons and 5 introns, and a high degree of homology has been found between these genes (93,94,103,104). In particular, exons 2, 5 and 6 have sequence homologies of 90 to 100%. Different motifs, expected to play a role in cholesterol 7α-hydroxylase gene expression, are clustered and partly overlapping each other in the promoter region of these genes (93,94,103,104). The rat gene, present in a single copy within the genome, does not share location of introns with any other cytochrome P-450 genes (92). The human cholesterol 7α-hydroxylase gene, also present as a single copy, has been mapped to chromosome 8q11-q12 (93). Genetic polymorphisms were identified in the coding region of the human gene (106) and in the 3'- and 5'-flanking regions and introns (93). Different animal studies also found linkage between cholesterol levels and cholesterol 7α-hydroxylase (22,23,107). It is tempting to speculate that polymorphisms in the cholesterol 7α-hydroxylase gene may explain the differences in bile acid synthetic capacity seen in humans (16) and animals (18).

The 5'-flanking region of the human cholesterol 7α-hydroxylase gene has been characterized (93,103,104,108). The sequence revealed many conserved

consensus sequence motifs for liver-enriched transcription factors which are conserved. The first 400 nucleotides appear to contain the fundamental elements required for basal expression, e.g. a TATA box at position -30, a modified CAAT box at position -92 which can bind C/EBP, four potential hepatocyte nuclear factor 3 (HNF-3) recognition sites at nucleotides -316, -288, -255, and -79 and other liver-specific enhancer elements for HNF-4 and COUP-TFII at -144 and for HNF-1 at -52 (93,103,104,108,109). The HNF-3 sites and the HNF-4 sites were found to be functionally active, implicating these latter sites are essential for cell-specific enhancement of cholesterol 7α-hydroxylase promoter activity (108,109). Furthermore, a functional binding site (-197 to –173) for a ubiquitous transcription factor was found. Deletion of this site resulted in an increased activity of the human cholesterol 7α-hydroxylase. The role of this factor in regulation is interesting to elucidate. Besides this, it is recently proposed that a number of regulatory elements responsive to many effectors such as phorbol esters and different hormones may reside in the human promoter from –298 to +24 (110). In rat a liver-specific enhancer was also located 7 kb upstream of the transcriptional initiation site (111). Homology between promoter sequences of human and rat, specifically within the region of -432 and -220, which appears to confer cell-specificity of human cholesterol 7α-hydroxylase, is limited to approximately 50%. However, within the first 250 bp 5'-flanking region sequence identity is approximately 75% among rat, hamster and human (91-94,103,112). Functional analysis of the rat cholesterol 7α-hydroxylase promoter in rat hepatocytes and $HepG_2$ cells showed that the major transcription-activating region is located in the proximal 145 nucleotides (112-117). This sequence element contains putative recognition sequences for the transcription factors C/EBP, HNF-3 and HNF-4, a basic transcription element (BTE), and the orphan nuclear receptor COUP-TFII. In addition, a number of DNA-sequences related to physiological regulatory processes, i.e. regulation by bile acids, hormones, (oxy)sterols, retinoids and diurnal rhythm have been identified (112-126), which will be discussed below. The variance in the regulatory elements present within the promoter regions of different species, suggests that regulation at the DNA level may show species-related differences.

3.2 Sterol 27-hydroxylase

As discussed above, the importance of sterol 27-hydroxylase as a site of regulation in the bile acid synthetic pathway may have been underestimated in the past. Recent

data indicate that the enzyme is modulated by different physiological effectors. The enzyme is a unique member of the cytochrome P-450 superfamily (CYP27), which catalyzes the 27-hydroxylation of various intermediates in the neutral pathway and of cholesterol, as the first step in the acidic or alternative pathway (28,30). It is localized exclusively in the mitochondrial inner membrane. After initial characterization and purification to near homogeneity by Okuda and coworkers (rat) and Wikvall and Dahlbäck (rabbit), the enzyme from rat and rabbit liver mitochondria were purified by Okuda et al (127) and Russell and coworkers (128). The cDNA for rabbit sterol 27-hydroxylase was cloned and shown to encode 535 amino acid residues, of which to first 36 amino acids consisted of the mitochondrial signal sequence (128). Later, the rat and human sterol 27-hydroxylase cDNAs have been isolated and shown to encode proteins with a molecular weight of 57 kD after cleavage of mitochondrial-type signal sequence. Large quantities of the human sterol 27-hydroxylase were purified after expression of this enzyme in *E. coli* for the further catalytic characterization (50). The mRNA has a relatively short 3' UTR, within which the sequences related to the instability of the cholesterol 7α-hydroxylase mRNA are virtually absent, and it appears to be transcribed from a single-copy gene (128-132).

Multiple mRNAs have been detected for sterol 27-hydroxylase of 2.4 kb and 2.1 kb in rat and 2.2 kb and 1.8 kb in human (129-131). The liver only exhibits expression of the longer mRNA species, but both are present in human fibroblasts and in rat ovaries, in the latter after stimulation with gonadotropin (129-131). The mRNA for sterol 27-hydroxylase showed an apparent half-life of approximately 13 hours, indicative of a much slower decline than cholesterol 7α-hydroxylase in cultured rat hepatocytes (133). The human sterol 27-hydroxylase gene has been isolated, and was shown to span a length of 18.6 kb, consisting of nine exons and eight introns (132,134). It is localized to the q33-qter interval of human chromosome 2 (132). The promoter region appeared GC-rich and contained putative recognition sites for transcription factor SP-1 and HNF-1 (134). Mutations in the sterol 27-hydroxylase gene underlie the sterol storage disorder cerebrotendinous xanthomatosis (CTX) (132,134-143).

Sterol 27-hydroxylase activity and mRNA are observed in several cell-types including fibroblasts, endothelial cells, artherosclerotic plaques and macrophages (144-149), and in tissues as liver, brain, adrenals, duodenum, ovary, kidney, and lung (128-131), indicating that the activity of this enzyme is not restricted to bile acid synthesis. Recently, a novel oxidative mechanism for elimination of intracellular cholesterol from cells of the vessel wall was put forward, in which cholesterol,

following conversion into 27-oxygenated products by sterol 27-hydroxylase, can be directed to the liver. The intermediates are subsequently converted into bile acids (145-147). *In vitro* studies demonstrated an interplay between HDL-mediated reverse cholesterol transport and the sterol 27-hydroxylase-dependent transport of cholesterol metabolites (150,151). The relative importance of the two different mechanisms seems to be different in the different species (150) and warrants further investigation.

It has been postulated that 27-hydroxylase is of importance for the overall regulation of cholesterol biosynthesis (28,152), since oxysterols are known to be potent regulators of the rate-limiting enzyme in cholesterol synthesis HMG-CoA reductase and of expression of the LDL receptor (153-155). The product of sterol 27-hydroxylase, 27-hydroxycholesterol, is a potent inhibitor of HMG-CoA reductase in fibroblasts (156,157). It is selectively formed from LDL cholesterol in these cells, i.e. no other biologically active oxysterols, such as 7α-hydroxycholesterol and 24- or 25-hydroxycholesterol, were found (157). Recent data indicate that further metabolites such as 7α, 27-hydroxylated intermediates, as present in the formation of bile acids, are the most potent inhibitors of HMG-CoA reductase (158). In addition, mRNA expression for the enzyme in different tissues could be correlated to cholesterol synthetic capacity (128,129) and 27-hydroxycholesterol may, therefore, have an effect on lipoprotein uptake by down-regulation of LDL receptor activity (155,156,159). Further evidence for a regulatory role of sterol 27-hydroxylase is derived from animal and human studies. Baboons showing a low response in elevation of plasma LDL to an atherosclerotic diet, supplemented with high cholesterol and fat, have markedly higher plasma 27-hydroxycholesterol and hepatic and extrahepatic 27-hydroxylase protein, enzyme activity and mRNA levels than high-responders. After several weeks on the diet plasma LDL cholesterol concentrations were negatively correlated with plasma 27-hydroxycholesterol concentrations and hepatic and extrahepatic sterol 27-hydroxylase protein, enzyme activity and mRNA levels (160-162). Patients suffering from cerebrotendinous xanthomatosis (CTX), resulting from a molecular defect in sterol 27-hydroxylase (132,134,135), have an uncontroled endogenous cholesterol synthesis and an abnormal cholesterol metabolism leading to build-up of abnormal levels of cholesterol and cholestanol in tissues and urinary and biliary excretion of C27-steroid derivatives (163). They exhibit gallstone disease, most probably caused by decreased levels of chenodeoxycholic acid synthesis (164). Despite normal or low levels of circulating cholesterol, patients with CTX are predisposed to develop

premature atherosclerosis (163). However, in mice with disrupted sterol 27-hydroxylase gene no CTX-related pathological abnormalities were observed but the lack of this enzyme has a more dramatic effect on bile acid synthesis in mice than in humans (49). The difference between these species is probably due to a compensatory mechanism is humans (165), which is characterized by activation of the 25-hydroxylase pathway and leads to formation of cholic acid.

The above data suggest that sterol 27-hydroxylase may act as an anti-atherosclerotic enzyme in the vessel wall by removing excess of cholesterol. It also provides a mechanism for prevention of accumulation of intracellular cholesterol by down-regulation of LDL receptor and HMG-CoA reductase expression. In addition, in the liver it may be involved in regulation of the amount of cholesterol to be converted into the bile acids.

3.3 Oxysterol 7α-hydroxylase

The finding that mice lacking the cholesterol 7α-hydroxylase gene are still capable to synthezise 7α-hydroxylated bile acids suggested another 7α-hydroxylase enzyme which has been described as oxysterol 7α-hydroxylase (73). In these cholesterol 7α-hydroxylase -/- mice oxysterol 7α-hydroxylase becomes important during neonatal life.

A cDNA has been isolated from mouse brain (62). The protein encoded by this enzyme is involved in the 7α-hydroxylation of 25- and 27-hydroxycholesterol (61,166). The fact that this enzyme can use oxysterols as substrate, suggest it's important role in regulation of oxysterol levels by using them as substrate, and thereby inactivating these important physiological regulators of cholesterol homeostasis (167). Such a mechanism would explain the unique property of the human liver to resist down-regulation of the LDL-receptor (168). Furthermore, this enzyme (called as CYP7B) is also active against dehydroepiandrosterone and pregnenolone (169). However, it is not yet very clear whether the enzyme involved in 7α-hydroxylation of 27-hydroxycholesterol and dehydroepiandrosterone is the same or involves at least 2 probably closely related enzymes (170). The encoded mouse protein shows a 39% homology with cholesterol 7α-hydroxylase. So far, only limited data is available about regulation of oxysterol 7α-hydroxylase. In mice, dietary cholesterol or colestipol did not have any effect on enzyme activity, mRNA and protein levels, whereas bile acids only modestly decreased the expression (61).

These results indicate that cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase are regulated differently.

Recently, Setchell *et al* (171) identified a mutation in the oxysterol 7α-hydroxylase gene in a neonate having severe cholestasis, cirrhosis, and liver synthetic failure. However, the cholesterol 7α-hydroxylase gene in this patient was normal. The mutation resulted in a truncated enzymatically inactive oxysterol 7α-hydroxylase protein. This finding indicates the quantitative importance of the acidic pathway in early human life.

3.4 Sterol 12α-hydroxylase

Little is known about regulation of other enzymes in the bile acid synthetic pathway and their importance for modulation of total bile acid synthesis (24,26,27). The only other enzyme that we would like to mention here is sterol 12α-hydroxylase (CYP8B1). Being the branching point within bile acid synthesis, the enzyme - as well as sterol 27-hydroxylase - is involved in the determination of the cholic:chenodeoxycholic ratio, and hence the relative hydrophobicity of the bile acid pool in man and rat. In the latter species, chenodeoxycholic acid is readily converted to the hydrophilic β-muricholic acid, both *in vivo* and *in vitro*. A strong correlation between 12α-hydroxylase activity and bile acid composition has been found in hamsters (172), although such a correlation is not evident in humans (173).

The sterol 12α-hydroxylase has been purified from rabbit liver, and is shown to be a microsomal cytochrome P-450 (174). The cDNA of rabbit sterol 12α-hydroxylase was cloned encoding a peptide of 500 amino acids, corresponding to a molecular mass of 57 kD. Northern-blotting showed that the enzyme is exclusively expressed in the liver having a size of 2.9 kb (175). Recently, the human and mouse sterol 12α-hydroxylase cDNA were cloned. Suprisingly, the genomic DNA from both species was found to lack introns (176). The mRNA for sterol 12α-hydroxylase identified from human liver has a size of 3.9 kb, whereas the sizes of the mouse liver RNA displayed a major band of 2.2-2.4 kb and a weaker band of 3.9 kb (176). With regard to regulatory processes affecting this enzyme, limited data is available (24,26,27). Biliary diversion and cholestyramine treatment increase the specific activity in rat (177) and man (178). Recently it was shown that also mRNA levels are increased in rat (179) after biliary diversion. Administration of 17α-ethinylestradiol to bile diverted rats resulted in a marked decline in mRNA levels of sterol 12α-hydroxylase (179). Furthermore, the enzyme is suppressed by chenodeoxycholic

acid treatment in rat and in humans (180,181), and by thyroxine in rat (182,183). Stimulation of 12α-hydroxylase activity is observed in rats with streptozotocin-induced diabetes (174,184,185), with a subsequent normalization by insulin treatment (185). Starvation markedly elevated the activity of sterol 12α-hydroxylase in mice (175,176) and rats (174,184,175). In contrast, starvation of rabbits had little or no stimulatory effects on enzyme acitivity as well as mRNA levels of sterol 12α-hydroxylase (175).

Table 1. Regulation of cholesterol 7α-hydroxylase

Treatment	Activity	Protein	mRNA	Transcription
Bile acids	↓	↓	↓	↓
Interruption of EHC	↑	↑	↑	↑
Diurnal rhythm				
Dark	↑	↑	↑	↑
Light	↓	↓	↓	↓
Glucocorticoids				
High dose	↓	↓	↓	?
Low dose	↑	?	↑	↑
Thyroid hormone	↑	↑	↑	↑
Insulin	↓	?	↓	↓
Starvation	↓	↓	↓	?
Cholesterol	↑	↑	↑	↑
Cholesterol synthesis inhibitors	↓	↓	↓	↓
Mevalonate	↑	?	↑	↑

Adapted from ref 26.

4. REGULATORY PROCESSES IN BILE ACID SYNTHESIS

A summary of the major effectors and processes regulating cholesterol 7α-hydroxylase is given in Table 1.

Most of our knowledge regarding regulation of bile acid synthesis has been gathered in the rat. Due to the absence of a gall bladder, and the presence of an unusually long small intestine, these animals exhibit a high capacity to form bile acids. Furthermore, differences in the bile acid composition exist among species (4). These anatomical and biochemical differences have to be kept in mind, when extrapolating results obtained in these animals to other species. Nevertheless, most animals share the same rate-limiting steps in bile acid biosynthesis, and with obvious exceptions many forms of regulation of the pathway, known to date, have been recognized over a wide range of different species.

4.1 Bile acid-induced feedback regulation

According to current concepts, the most important way by which bile acid synthesis is regulated, is by the flux of bile acids returning to the liver via the enterohepatic circulation (EHC). This hypothesis was initially based on experiments of Thompson and Vars (186), Eriksson (187) and others (81,177,188,189), showing a several-fold stimulation of bile acid synthesis in rat after complete biliary diversion (CBD), or by administration of anion-exchange copolymers that bind bile acids in the small intestine and thereby prevent their uptake and feedback regulation. Further evidence was provided by administration of bile acids to CBD-rats, either intraduodenally or intravenously, leading to normalization of bile acid synthetic capacity (81,188,190). Shefer *et al.* (81) subsequently showed that administration of taurocholate inhibited the conversion of radiolabeled acetate, mevalonate, and cholesterol but not of 7α-hydroxycholesterol, indicating that the enzyme responsible for the first and rate-limiting step in the conversion of cholesterol to bile acids, cholesterol 7α-hydroxylase, was the regulatory point. This was also evidenced by experiments in which biliary drainage or treatment with the bile acid sequestrant cholestyramine were found to stimulate cholesterol 7α-hydroxylase activity 5 to 8-fold, whereas several other enzymes involved in bile acid synthesis were affected to a lesser extent or not at all (177,191). Princen and colleagues were the first to show that addition of physiological concentrations of bile acids to the culture medium of primary pig

hepatocytes resulted in a strong and concentration-dependent decrease of bile acid synthesis and cholesterol 7α-hydroxylase activity (192,193). Further resolution as to the level of regulation and the molecular mechanism involved had to await the isolation of cDNA and antibody probes for cholesterol 7α-hydroxylase. It has been established now that bile acid feeding reduces and bile diversion or cholestyramine treatment increases *in vivo* levels of cholesterol 7α-hydroxylase enzyme activity, protein mass, mRNA, and gene transcription (85,87,88,90,194,195). Similarly, downregulation of bile acid synthesis by bile acids in cultured rat hepatocytes was reported to be modulated at these molecular levels (97,112,115,193,196). A similar regulation of cholesterol 7α-hydroxylase as in rat occured in humans (34,35,197-199).

While regulation studies have focused mainly on cholesterol 7α-hydroxylase as a major point of regulation by bile acids, Twisk *et al.* showed recently that sterol 27-hydroxylase is also suppressed at the level of enzyme activity, mRNA and gene transcription by physiological concentrations of different bile acids in cultured rat hepatocytes (119,133). These findings were confirmed later in part by others, showing suppression of the sterol 27-hydroxylase mRNA by taurocholate but remarkedly not of the protein and enzyme activity (200). *In vivo* experiments with rats further supported the view that bile acids returning to the liver via the EHC may regulate sterol 27-hydroxylase. Treatment of rats with bile acid-binding resins induced a 2-to 3-fold increase in sterol 27-hydroxylase activity, mRNA and gene expression (201,202). Furthermore, in bile fistulated rats sterol 27-hydroxylase enzyme activity and mRNA level showed a similar increase (179). Conversely, feeding rats a diet containing hydrophobic bile acids suppressed the activity, mRNA and transcriptional activity of sterol 27-hydroxylase (202). In these studies the responses of sterol 27-hydroxylase to manipulation of the bile acid pool were less prominent than observed with cholesterol 7α-hydroxylase. These results suggest that co-ordinate suppression of both enzymes may efficiently lead to homeostatic regulation of bile acid synthesis. However, in other *in vivo* studies in rat and rabbit, treatment with cholestyramine or bile acids or bile fistulation did not show significant effects on sterol 27-hydroxylase (128,203-207). Thus, there appears to be inconsistency with respect to regulation of sterol 27-hydroxylase by bile acids. Whether 27-hydroxylase in man is modulated by bile acids is not known: conflicting indirect data have been reported. In hypercholesterolemic patients treatment with cholestyramine resulted in normal or increased levels of 27-hydroxycholesterol in

plasma (208), and Axelson and Sjövall found an increase in one intermediate (7α-hydroxy-3-oxo-4-cholestenoic acid) potentially representative for involvement of the 27-hydroxylase pathway in man treated with cholestyramine, but not of two other intermediates (42).

4.1.1 Factors involved

There was still concern, however, whether bile acids must be regarded as the actual regulators of cholesterol 7α-hydroxylase, or if effects were exerted indirectly. Initial failure to detect an effect of bile acids on bile acid synthesis in rat hepatocytes in suspension (209), or in monolayer culture (210,211), even in concentrations greatly exceeding those found in portal blood of rats, seriously challenged the concept of feedback regulation. Moreover, in later studies no relationship was found between portal bile acid concentrations and cholesterol 7α-hydroxylase activity after feeding of different bile acids or cholestyramine to rats (212), the latter in contrast to an earlier report (213). It was suggested that an intact EHC be a prerequisite for bile acid-induced feedback regulation, and that other intestinal factors be the real repressors of cholesterol 7α-hydroxylase (214). These factors would be dependent on bile acid flux for their release from the intestine (215,216). However, Princen and colleagues (97,119,192,193) and others (196) demonstrated that addition of physiological concentrations of bile acids to the culture medium of pig and rat hepatocytes, respectively, resulted in a strong decrease of bile acid synthesis and cholesterol 7α-hydroxylase activity, mRNA and gene transcription, indicative of a direct effect of these compounds on the hepatocyte. This was confirmed recently *in vivo* in hamsters, in which the availability of bile salts within the hepatocyte was manipulated by overexpression of the cholesterol 7α-hydroxylase gene, obstruction of the common bile duct and intravenous infusion of taurocholate (217).

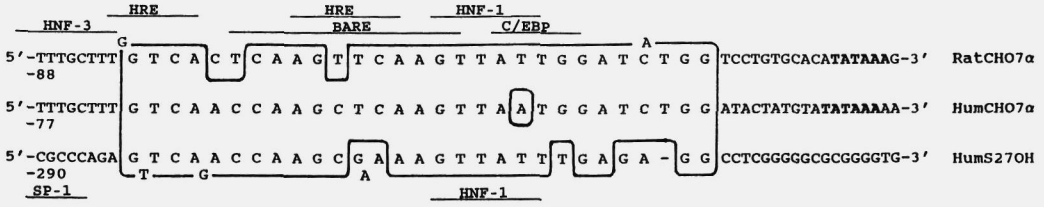
Another working hypothesis was based upon the observation of similar induction of both HMG-CoA reductase and cholesterol 7α-hydroxylase as a result of biliary diversion or, alternatively, co-ordinate suppression of the two enzymes after bile acid feeding (24,25,218,219 and others). The possibility that cholesterol 7α-hydroxylase might be regulated by the flux of newly synthesized cholesterol in this way, was addressed by Pandak *et al.* (220). Experiments, in which taurocholate infusion was combined with constant substrate supply, in the form of mevalonate, showed sustained inhibition of cholesterol 7α-hydroxylase, indicating this not to be a likely option (220).

4.1.2 Molecular mechanism

With respect to the molecular mechanism of regulation, there are two models proposed which may account for the transcriptional inhibition of cholesterol 7α-hydroxylase by bile acids, a direct or an indirect model. On the one hand, a direct effect may be established by binding of bile acids to a putative bile acid receptor, which after entering the nucleus, interacts with the cholesterol 7α-hydroxylase promoter, in analogy with regulation of gene expression by members of the steroid-thyroid receptor superfamily. Using functional analysis in transient expression assays with promoter-reporter constructs in primary rat hepatocytes and $HepG_2$ cells, Hoekman *et al.*(112), and Chiang and Stroup (115) have both identified a bile acid-responsive element (BARE) in the 5' flanking region, which is located in the proximal promoter between -49 and -79 bp. Within this region a direct repeat TCAAGTTCAAGT is present, which is the binding site of a 57 kD nuclear protein, possibly acting as a negative transcription regulator. Low specificity was shown, however, with respect to binding of the direct repeat to factors from various rat nuclear extracts from control, bile acid-fed, or cholestyramine-treated rats (115). Other putative regulatory sequences within this region are imperfect repeats of hormone receptor elements (HREs), together forming a region responsive to certain hormones (114) (Figure 4). The superficial resemblance of bile acids to steroids could provoke an interaction with these HREs via general hormone receptors (see below). However, deletion of this BARE enhanced the reporter activity in transient transfection experiments in Hep G2 cells but did not completely abolish the bile acid response. This is due to a second BARE located in a conserved region between nt -149 and -128 identified by Stroup *et al.* (212). The BARE-II shares an identical AGTTCAAG core sequence with BARE-I. How these two BAREs interact and regulate transcription has to be further investigated.

The sterol 27-hydroxylase promoter has not been functionally characterized. Promoter sequences of the human sterol 27-hydroxylase have been published (134), however, and a region between -254 and -280 was noticed (119,133), which revealed a high degree of homology to an element in the -49 to -79 region of the rat cholesterol 7α-hydroxylase promoter (112,113). This region harbours two putative HRE-sequences, in part overlapping a consensus sequence for HNF-1, indicating a similar topology of several putative binding sequences, which have been conserved between the promoters of two different enzymes (Figure 4). This may explain the co-ordinate transcriptional regulation of both enzymes by similar bile acids in cultured rat hepatocytes (119,133,200).

A



B

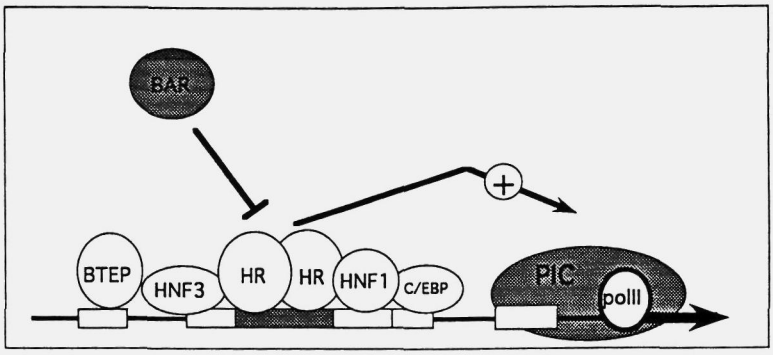


Fig. 4 Model of the transcriptional regulation of cholesterol 7α-hydroxylase gene expression.
A. Sequence homology between a distinct promoter region of the human sterol 27-hydroxylase promoter (sequence from 134) and of the region containing the bile acid regulatory element in rat (112,115) and human (120). B. Representation of transcription factors binding to proximal cis-acting elements (boxes) as shown in A and regulating CYP7 transcription. PIC, preinitiation complex, consisting of polymerase II (pol II) and basic transcription factors (not shown). C/EBP, CAAT-element binding protein; HNF-1 and HNF-3, hepatic nuclear factors 1 and 3, HR, hormone receptor; BTEP, basic transcription element binding protein (element between -89 to -98, not shown). The putative Bile Acid Receptor (BAR) interacts either with the Hormone Receptors or binds to motifs within the HRE region (indicated by arrow). CYP7A transcription is subsequently down-regulated through interactions between the modulated basal complex and the preinitiation complex, indicated by arrow/stop (from Hoekman,114).

On the other hand, there are indications from other studies that bile acids may be largely excluded from hepatocyte nuclei and no cytosolic bile acid binding protein with characteristics of a steroid hormone receptor has been identified (222-225). In addition, in band-shift assays involving the above mentioned BARE(I) and liver nuclear extracts from control and deoxycholic acid-treated rats, no binding was found with the latter extracts (115). These data suggested that bile acids may suppress 7α-

hydroxylase gene expression indirectly, i.e. by binding to a bile acid-binding protein, which in turn interacts with and prevents the stimulatory action of hepatic transcription factors.

Alternatively, bile acids have been proposed to modulate the signal transduction pathway involving protein kinase C (PKC) in proportion to their hydrofobicity index (226), ultimately leading to modifications of a *trans*-acting factor required for cholesterol 7α-hydroxylase transcription (120,227). It was shown that bile acids were able to activate PKCα and δ (226,228) and the repressive effect of bile acids on cholesterol 7α-hydroxylase could be blocked by specific PKC inhibitors (227). These and other experiments (229) suggest that bile acids repress cholesterol 7α-hydroxylase gene expression by activation of PKC. This was supported by transient transfection experiments with HepG2 cells, in which treatment with the phorbolester PMA resulted in a time-dependent inhibition of the human (110) and rat (120) cholesterol 7α-hydroxylase promoter activity. It is possible that PKC is activated by bile acids via diacylglycerol, since a correlation was found between bile acid structure and diacylglycerol formation (228). However, further experiments are necessary to unravel the mechanism by which bile acids activate PKC and how the signal is transduced to the nucleus in order to regulate cholesterol 7α-hydroxylase gene transcription.

4.1.3 Levels of regulation

The inhibitory effect of bile acids on cholesterol 7α-hydroxylase activity and mRNA, as observed in cultured rat hepatocytes, can not solely be explained by transcriptional regulation of cholesterol 7α-hydroxylase, suggesting that bile acids affect both the transcriptional and the post-transcriptional i.e. mRNA level (97,112,119). Further support for this view is provided by the apparent discrepancy between lobular distribution of cholesterol 7α-hydroxylase activity and mRNA as compared with the relative levels of gene transcription within the lobulus; pericentral to periportal levels differ twofold (201). The predominant pericentral localization of cholesterol 7α-hydroxylase has been attributed to the concentration gradient of bile acids over the lobulus, which is high in the periportal region and low in the pericentral zone (see below). In line with the assumption of differential regulation at transcriptional and mRNA level, analysis of the primary and secondary structure of the 3'non-coding region of the cholesterol 7α-hydroxylase mRNA has revealed the presence of AU-rich sequences (98), shown to be involved in destabilization of

various mRNAs (99,100). Agellon *et al.* (230) demonstrated that the 3' non-coding region of the mouse cholesterol 7α-hydroxylase mRNA contained elements responsive to post-transcriptional regulation by bile acids. Interestingly, others showed data, suggesting that a posttranslational mechanism may also be active in addition to the other levels of control in regulation of the cholesterol 7α-hydroxylase activity in humans treated with cholestyramine (231) and in rats fed bile acids (232).

4.1.4 Bile acid structure

With regard to a functional structure of a bile acid in terms of potency to inhibit bile acid synthesis and cholesterol 7α-hydroxylase, it has been postulated that repressional activity is directly correlated to hydrophobicity of a given bile acid (196,218). After feeding different bile acids to rats with an intact EHC, it was observed that relatively hydrophilic bile acids (ursocholate, ursodeoxycholate, hyocholate, hyodeoxylate) did not affect cholesterol 7α-hydroxylase activity. However, more hydrophobic bile acids (cholate, chenodeoxycholate, deoxycholate) inhibited cholesterol 7α-hydroxylase and HMG-CoA reductase activities in order of increasing hydrophobicity (218). In addition, other views exist, reporting taurocholate not active, whereas taurodeoxycholate and taurolithocholate were strong inhibitors of bile acid synthesis in rat (233) and rabbit (234). It was suggested that primary bile acids first have to be converted into secondary bile acids by intestinal bacteria in order to become regulatory (233,234). This view was supported by experiments, in which intravenous infusion of taurocholate did not show feedback regulation, in contrast to intraduodenal administration of the compound (235). Heuman *et al.* proved this not to be the case (236). Alternatively, specifically monohydroxy bile acids derived from either intestinal or hepatic sources, were reported to effectively down-regulate bile acid synthesis in the rabbit, indicating that the hydroxylation status is important for the inhibitory potency (234).

The 7β-hydroxy epimers of both cholic acid and chenodeoxycholic acid showed little or no ability to regulate the expression of cholesterol 7α-hydroxylase in bile-fistulated rats (237,238) and in man (198). Epimerization of the 7-hydroxygroup, yielding such drastic differences in effect on this enzyme, suggests that the specific three-dimensional structure of a bile acid may also be important in determining the ability to regulate, rather than the hydrophobicity alone. The latter is supported by results obtained with cultured pig and rat hepatocytes, in which cholate, hyodeoxycholate, chenodeoxycholate and deoxycholate showed equal suppressive

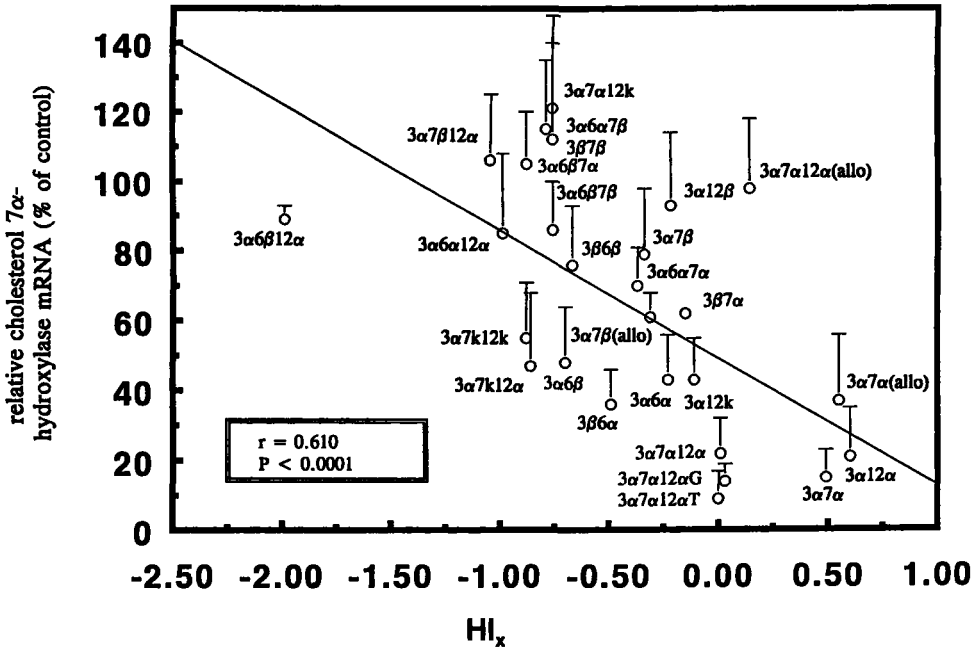


Fig. 5 Effect of different bile acids on cholesterol 7α-hydroxylase mRNA levels compared to their hydrophobicity index.
Cholesterol 7α-hydroxylase mRNA is plotted versus HI_x (hydrophobicity index). Reprinted with permission from Twisk J, Hoekman MFM, Muller LM, Iida T, Tamaru T, IJzerman A, Mager WH, Princen HMG. Structural aspects of bile acids involved in the regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Eur J Biochem 1995; 228: 596-604.

effects on bile acid synthesis and cholesterol 7α-hydroxylase, although differing significantly in hydrophobicity index (97,192,193). In a large study with primary rat hepatocytes, incubated with 27 bile acids differing highly in hydrophobicity index, and number, position, and orientation (α,β) of the hydroxylgroups present on the steroid backbone, a mild correlation between potency of down-regulation of cholesterol 7α-hydroxylase and the hydrophobicity index (R= 0.61) was found (Figure 5, 119). Princen and colleagues postulated, therefore, that the hydroxyl groups present on the steroid backbone may form a hydrophilic microenvironment within an otherwise hydrophobic molecule, provided that the different hydroxyl groups are located in the proper position and orientation (Figure 6). Creation of such an environment may be a prerequisite for binding to a factor involved in either direct or indirect interaction with regulatory sequences within the cholesterol 7α-hydroxylase and sterol 27-hydroxylase promoter (119).

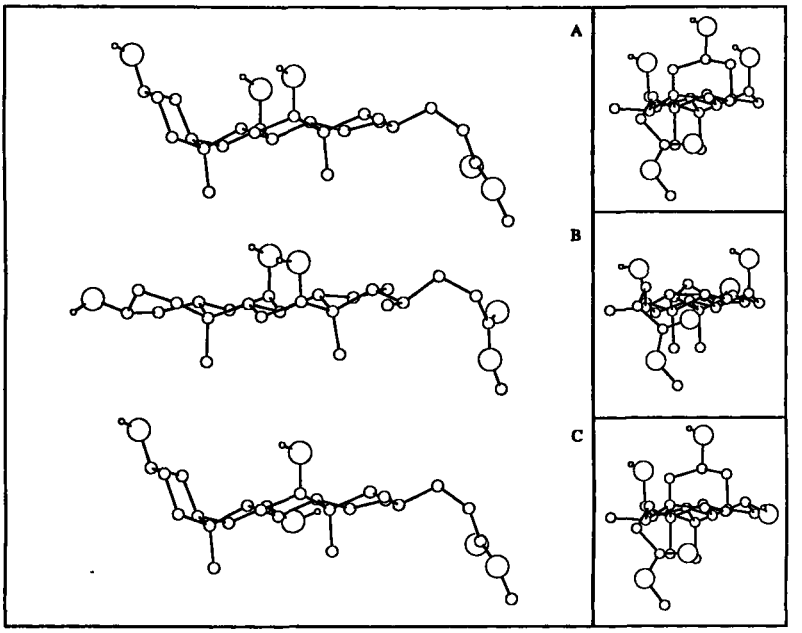


Fig. 6 Three-dimensional structure of cholate (A) compared to that of allo-cholate (B) and ursocholate (C).
The three-dimensional depiction of cholate is based on the crystallographical studies of methylcholate. Small circles, hydrogen atoms (shown only when connected to oxygen atoms); intermediate circles, carbon atoms; large circles, oxygen atoms. Left panel, cholate (A), allo-cholate (B) and ursocholate (C); right panel, A-C as seen from the side-chain towards the steroid nucleus. Reprinted with permission from Twisk J, Hoekman MFM, Muller LM, Iida T, Tamaru T, IJzerman A, Mager WH, Princen HMG. Structural aspects of bile acids involved in the regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Eur J Biochem 1995; 228: 596-604.

4.2 Diurnal regulation

Cholesterol 7α-hydroxylase follows a circadian rhythm with an amplitude of two- to threefold synchronous with that of HMG-CoA reductase. In rat, mouse and rabbit the enzymes show a peak in protein, enzyme activity and mRNA at midnight, parallel to the nocturnal feeding-behaviour of most rodents (75,85,88,90,98,239). Recently, it was shown that the transcription rate of cholesterol 7α-hydroxylase follows the same circadian cycle with the highest expression at midnight (240). No sexual difference was observed in the diurnal rhythm (90,98). Glucocorticoids have been implicated to play a role in the control of the diurnal rhythm of cholesterol 7α-hydroxylase and

HMG-CoA reductase (75,241). Because the diurnal rhythm is synchronized by the photoperiod, it is plausible that the circadian changes in cholesterol 7α-hydroxylase gene expression may be initially precipitated by neuroendocrine factors. Additionally, the observed variations in cholesterol 7α-hydroxylase activity levels follow the circadian pattern of serum concentrations of glucocorticoids with a lag of approximately 3 to 4 hours (241). Bilateral adrenalectomy or suppressing the secretion of adrenocorticotrophic hormone (ACTH) abolished diurnal changes (75,241), and glucocorticoids induced cholesterol 7α-hydroxylase in cultured rat hepatocytes (242,243), suggesting that glucocorticoids play an important role in this phenomenon.

On the other hand, the diurnal rhythm of cholesterol 7α-hydroxylase parallels the expression of the transcription factor albumin element D binding protein (DBP) (244), a member of the PAR family, which in its turn is a subfamily of the basic leucine zipper proteins (bZIP). Several DBP-responsive elements have been found in the promotor region of cholesterol 7α-hydroxylase (121,122). Co-transfection experiments showed stimulation of transcription from the 7α-hydroxylase promotor by DBP (121) and the human leukemia factor 43 (HLF43), which is also a member of the PAR subfamily (245). It has been suggested that DBP competes with C/EBPβ (LAP) to activate cholesterol 7α-hydroxylase gene transcription and that this competition determines the relative rates of basal versus diurnally regulated cholesterol 7α-hydroxylase gene transcription (122). In rats, the expression of DBP was also shown to be modulated by a change in the schedule of parenteral nutrition resulting in parallel changes in cholesterol 7α-hydroxylase transcription (246). Switching off the biological rhythm by destruction of the suprachiasmatic nucleus of the hypothalamus altered the diurnal rhythms of DBP and HMG-CoA reductase mRNA levels markedly (247). Addition of dexamethasone represses accumulation of DBP mRNA and protein in rats, suggesting that glucocorticoids are not the direct mediators of the diurnal activity (244). Recent data indicate that sterol 27-hydroxylase activity may be also subject to diurnal regulation with the highest activity in the mid-dark phase, in parallel with cholesterol 7α-hydroxylase (202).

4.3 Hormonal regulation

Several hormones have been implicated to play an important role in regulation of bile acid biosynthesis, and especially of cholesterol 7α-hydroxylase, both *in vivo* and *in vitro* (reviewed some time ago, see references 25,26,75).

4.3.1 Glucocorticoids

A loss or reduction in cholesterol 7α-hydroxylase activity was shown *in vivo* in adrenalectomized rats, suggesting a role for glucocorticoids in positive regulation of bile acid synthesis. Indeed, administration of glucocorticoids to these animals led to partial restoration of cholesterol 7α-hydroxylase activity (75,241). Dexamethasone, a synthetic glucocorticoid, has also been shown to increase bile acid synthesis and enzyme activity, mRNA levels and transcriptional activity of cholesterol 7α-hydroxylase in primary cultures of rat hepatocytes (242,243), and gene transcription in transient transfection experiments with the rat promoter (114,120). Based on the latter study, two glucocorticoid responsive elements (GREs) have been identified in the 7α-hydroxylase promoter (120). However, dexamethasone appears to have more general stimulatory effects on gene expression. Addition of the glucocorticoid to primary cultures of rat hepatocytes not only induced cholesterol 7α-hydroxylase gene transcription, but also transcription of genes coding for sterol 27-hydroxylase, ß-actin, glyceraldehyde-3-phosphate-dehydrogenase and albumin (114). Upregulation of sterol 27-hydroxylase by glucocorticoids was recently also reported by others (200). However, in the rat hepatoma cell line L35, dexamethasone could only have a stimulatory effect on cholesterol 7α-hydroxylase gene transcription in presence of sulfhydryl reducing agents (248). Furthermore, *in vivo* treatment of rats with high doses of dexamethasone led to depressed cholesterol 7α-hydroxylase enzyme and mRNA levels (85,88). In addition, neonatal rat pups injected with dexamethasone showed higher plasma cholesterol levels, as well as a reduced cholesterol 7α-hydroxylase activity. The latter finding was explained by an increase in ileal bile acid absorption (249). In addition, experiments using the human cholesterol 7α-hydroxylase promoter containing two GREs demonstrated reduced gene promoter activity by dexamethasone (110). All together these findings indicate that the hormone may affect bile acid synthesis in different ways.

4.3.2 Thyroid hormone

Thyroid hormones, which are important physiological regulators of cholesterol metabolism at various levels, stimulate bile acid synthesis and cholesterol 7α-hydroxylase activity *in vivo* (75,250). Thyroidectomy and hypophysectomy decreased cholesterol 7α-hydroxylase activity (75), while subsequent administration of thyroid hormones in hypophysectomized rats led to a rapid increase in cholesterol 7α -hydroxylase mRNA (251,252). In another study by Rudling and co-workers (253),

normalisation of the enzyme activity was shown after growth hormone substitution. However, fecal excretion of bile acids in these rats were back to normal levels after administration of growth hormone in combination with thyroid hormone and cortisone. Simultaneous addition of dexamethasone and thyroid hormone to hepatocytes in culture was shown to have both positive and synergistic effects on cholesterol 7α-hydroxylase activity, mRNA and gene transcription (243). A synergistic effect in human hepatocytes was also found by Ellis *et al.* (254), however, an individual effect of these hormones were not found. Besides this, adrenalectomy together with thyroidectomy resulted in a decreased gene transcription of cholesterol 7α -hydroxylase. Whereas, neither adrenalectomy nor thyroidectomy alone affected transcriptional activity (255). These studies suggests cross-talk between these hormones. A study with patients with hypo- and hyperthyroidism also suggested no effect of thyroid hormone alone. In these patients no significant effect on serum 7α-hydroxy-4-cholestene-3-one after thyroid replacement or anti-thyroid treatment, respectively, was found (256). Transient transfection experiments in primary rat hepatocytes (112) and $HepG_2$ cells (110,257), revealed putative thyroid response elements (TREs) within the rat and human cholesterol 7α-hydroxylase promoter, although data are not consistent (120). Thyroid hormone has no effect on sterol 27-hydroxylase (183,200), but may increase the ratio of chenodeoxycholic acid to cholic acid, possibly by affecting sterol 12α-hydroxylase (182,183). An increased ratio was also found in hepatocytes incubated with a combination of thyroid hormone and dexamethasone (254). The hormone is also involved in maturation of bile acid metabolism in the neonatal phase (258).

4.3.3 Insulin and glucagon

Hormones of the pancreas, insulin and glucagon, also have an effect on bile acid metabolism. *In vivo* studies in patients suffering from diabetes mellitus and in experimental diabetic animals show that the bile acid pool and bile acid secretion is increased, and that these parameters can return to normal levels after administration of insulin (259-261). The higher bile acid excretion was ascribed to an increased bile acid synthesis. Twisk *et al.* recently showed that addition of physiological concentrations of insulin to primary rat hepatocytes led to a decrease in both cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA levels and gene transcription (118). Transient expression experiments in cultured rat

hepatocytes and $HepG_2$ cells have revealed responsiveness to insulin within the proximal region of the cholesterol 7α-hydroxylase promotor (110,114,118,120).

Incubation of freshly isolated rat hepatocytes in suspension with glucagon or cAMP-analogues have been shown to increase bile acid synthesis (262,263), and transient expression experiments in $HepG_2$ cells revealed a positive responsiveness for cAMP (120). In contrast, in primary monolayer cultures of rat hepatocytes a down-regulation of cholesterol 7α-hydroxylase mRNA in response to either glucagon or cAMP has been reported (196,243). Additionally, cholesterol 7α-hydroxylase was found to be down-regulated *in vivo* by starvation, a state in which glucagon levels are elevated (98,264). In this situation thyroid levels are reduced, but this is not responsible for the decrease in cholesterol 7α-hydroxylase in rats (265). Sterol 27-hydroxylase is not regulated by cAMP in primary rat hepatocytes (200). Concerted actions of insulin, glucagon, cAMP and hydrocortison have been implicated in determining HMG-CoA reductase activity (266). Specifically, insulin has opposite effects on HMG-CoA reductase versus cholesterol 7α-hydroxylase and sterol 27-hydroxylase (118).

4.3.4 Sex hormones

Female sex hormones, estradiol and progesterone, are known to increase the incidence of cholesterol gallstones in humans (267) and an *in vivo* study in rat suggests a correlation between a decrease in bile acid synthesis and administration of female sex hormones (268). Administration of 17α-ethinylestradiol to rats with an intact enterohepatic circulation resulted in a decrease in bile acid pool size and a change in the composition of the pool without a change in bile acid synthesis. In contrast, in long-term bile-diverted rats, there is a clear reduction in bile acid synthesis due to 17α-ethinylestradiol treatment. In both intact and bile-diverted rats, 17α-ethinylestradiol decreased cholesterol 7α-hydroxylase enzyme activity (179,269,270), whereas mRNA levels were reduced only in bile-diverted animals (179). In the latter study, sterol 27-hydroxylase was not affected by 17α-ethinylestradiol (179). In the intact rat, in which bile acid synthesis was not affected, the suppression of the neutral pathway to bile acid synthesis is apparently compensated for by an increased flux of cholesterol via the acidic pathway, resulting in a change in the bile acid pool composition. In the bile-diverted rats sterol 12α-hydroxylase and lithocholic acid 6β-hydroxylase were also affected by 17α-ethinylestradiol. Therefore, the effects of 17α-ethinylestradiol further down-stream in

the synthetic cascade may also contribute to determining the ratios between the various end products of both bile acid synthetic pathways. Progesterone also showed suppressive effects on cholesterol 7α-hydroxylase activity in rats (269,270), hamster (271) and $HepG_2$ cells (272). However, not all studies are in agreement with regard to the effects of sex hormones on bile acid synthesis. Ovariectomized baboons fed a high cholesterol and high saturated fat diet showed increased activity of cholesterol 7α-hydroxylase upon treatment with estrogen, whereas progesterone did not show this effect (273). Comparable effects were found on sterol 27-hydroxylase in perimenopausal baboons (274). In addition, hepatic sterol 27-hydroxylase activity and mRNA were enhanced in baboons treated with estrogen or the combination of estrogen and progesterone, whereas progesterone alone had no effect (275). Progesterone and estradiol were also found to activate bile acid synthesis in primary cultures of rat hepatocytes (276). In similar studies, however, Princen *et al.* showed the absence of any effect of sex steroid hormones, pregnenolone, or the mineralocorticoid aldosterone, on bile acid synthesis and cholesterol 7α-hydroxylase (242). In human subjects treatment with estrogens for metastatic prostate cancer also failed to show an effect on cholesterol 7α-hydroxylase, while simultaneously stimulating expression of the LDL receptor and HMG-CoA reductase activity (277).

4.3.5 Miscellaneous

The vitamin A-derived all-trans retinoic acid and its analogues have been demonstrated to play an important role in the regulation of genes involved in lipid and cholesterol metabolism (e.g. 278-280). Gene transcription of rat cholesterol 7α-hydroxylase is also stimulated by all-trans retinoic acid or 9-cis retinoic acid, both in transient transfection experiments using $HepG_2$ cells (120,124) and in primary rat hepatocytes (114). Retinoids exert their effects via the nuclear retinoid receptors (RXR/RXR or RXR/RAR). The binding-site for RXR/RAR is located in the rat promoter region of cholesterol 7α-hydroxylase (120,124). Different other nuclear receptors which were shown to affect promoter activity of the cholesterol 7α-hydroxylase gene can form heterodimers with RXR i.e. LXR (126) and COUP-TFII (116). The cytokines interleukine-1 and tumor necrosis factor-α were found to decrease cholesterol 7α-hydroxylase strongly in control and cholestyramine-fed hamsters, indicative for down-regulation of the enzyme during the acute phase response (281).

4.4 Regulation by endogenous and exogeneous cholesterol

A regulatory role within bile acid synthesis has also been assigned to cholesterol, its precursors or its metabolites. Studies addressing this form of regulation can roughly be devided into three groups. 1) Those that manipulate the flow of newly-synthesized cholesterol or of intermediates derived from the cholesterol synthetic pathway, and 2) those that manipulate uptake of exogenous preformed cholesterol by the liver, i.e. in the form of lipoproteins. The question has further arisen whether 3) cholesterol itself or metabolites of cholesterol, such as oxysterols, may be regulatory in analogy with regulation of HMG-CoA reductase and LDL receptor gene expression.

One mode of regulation, disregarding the source of cholesterol and different from transcriptional events described so far, involves the level of saturation of the cholesterol 7α-hydroxylase enzyme by its substrate cholesterol and has been subject to discussion (282-284). However, Einarsson and colleagues showed that various treatments leading to strong fluctuations in cholesterol 7α-hydroxylase activity, had no or only minor effects on the saturation index of the enzyme in rat and human liver microsomes (197,198,284).

4.4.1 Newly-synthesized cholesterol and its precursors

Decreasing the flow of *de novo* synthesized cholesterol, by administration of HMG-CoA reductase inhibitors to short or long term bile-fistulated rats, has been shown to lead to a rapid decrease in cholesterol 7α-hydroxylase activity, mRNA and gene transcription (86,220,285,286). Simultaneous infusion with mevalonate prevented this down-regulation (285,286), but no further induction of cholesterol 7α-hydroxylase was found after infusion of mevalonate in fistulated rats (194,286). Infusion of mevalonate in control rats also stimulated cholesterol 7α-hydroxylase activity, mRNA and gene expression (89,285,286). Although it has been suggested that regulation may take place at different levels, i.e. at the enzyme, mRNA or transcriptional level (90,194,220,285), these data indicate a regulatory role for mevalonate-derived products or newly-synthesized cholesterol. Experiments in which inhibitors further downstream in the cholesterol synthetic pathway were employed, also showed down-regulation of cholesterol 7α-hydroxylase, which could not be overcome by simultaneous treatment with mevalonate. The inhibitors used and their targets were zaragozic acid A, an inhibitor of squalene synthase (287), and AY9944, an inhibitor of 7-dehydrocholesterol-Δ^7-reductase (288). Similar data were obtained

using cultured rat hepatocytes (289). From these data is can be concluded that intermediates in the cholesterol synthetic pathway do not act as regulators of cholesterol 7α-hydroxylase. Treatment of control rats with lovastatin did not affect cholesterol 7α-hydroxylase (290), indicating that the supply of cholesterol as regulator is sufficient, probably by the strong upregulation of HMG-CoA reductase in this species under these conditions. Similarly, in human taking HMG-CoA reductase inhibitors no changes in cholesterol 7α-hydroxylase activity and bile acid synthesis were found (199,291,292), and enough regulatory cholesterol may be available via LDL receptor-mediated uptake. Sterol 27-hydroxylase expression in cultured hepatocytes (200) and *in vivo* in rat (72) was not affected by a cholesterol synthesis inhibitor.

4.4.2 Pre-existent cholesterol

The role of dietary cholesterol in regulation of bile acid synthesis has also been assessed. In rats, parallel increases in cholesterol 7α-hydroxylase activity, mRNA and transcriptional activity were found upon feeding a cholesterol-rich diet (86-88,111,195, 219,293). This induction was also observed in several other species, i.e. in mouse (294), dog (295), certain nonhuman primates (296) and in man (197). However, not all animals show a similar response. No change was found in baboons (161) and in hamsters (297), and a study with African green monkeys showed down-regulation of cholesterol 7α-hydroxylase activity and mRNA in response to dietary cholesterol (298). A similar finding was made in rabbits fed a cholesterol diet (299). A low basal rate of bile acid synthesis and a different response of cholesterol 7α-hydroxylase expression to dietary cholesterol render the latter species, therefore, more sensitive to the hypercholesterolemic effects of cholesterol than e.g. rat (297). Genetic differences in bile acid synthetic capacity and expression of 7α-hydroxylase within species have also been reported and may explain the hypo- and hyperresponsive behaviour of animals in terms of elevation of plasma cholesterol levels in response to dietary cholesterol (18,299,300). However, a decreased bile acid synthesis does not ultimately lead to hypercholesterolemia as shown in mice lacking the cholesterol 7α-hydroxylase (301) or sterol 27-hydroxylase (49) gene. Some studies report induction of sterol 27-hydroxylase in response to a cholesterol challenge in hypo-responding baboons (161) and rabbits (205), indicating that the alternative route to bile acids may also be responsive to circulating cholesterol levels.

From studies described so far, it remains unclear whether cholesterol exerts its effect directly on cholesterol 7α-hydroxylase, or whether other factors may also contribute to the effects observed. Studies by Björkhem and coworkers (214,293,302) have led to suggest that dietary cholesterol may interact with bile acids in the intestine, leading to malabsorption and reduced potential for bile acid-induced feedback. In agreement with this hypothesis, intraveneously administered Intralipid enriched in cholesterol failed to stimulate bile acid synthesis (293,302). Although an attractive view, a later study showed that, while intestinal absorption of cholic acid is inhibited by dietary cholesterol, absorption of chenodeoxycholic acid in humans is not (292), indicating that some potent down-regulators of bile acid synthesis escape intestinal entrapment. Furthermore, administration of both cholesterol and bile acids to rats has been shown to up-regulate cholesterol 7α-hydroxylase, while HMG-CoA reductase was down-regulated (297,303) suggesting that dietary cholesterol does reach the liver, eliciting its multiple effects. Xu *et al.* suggested that the decrease in cholesterol 7α-hydroxylase on a cholesterol-rich diet in rabbits is also due to an indirect effect. Increase in the bile acid pool on a cholesterol-rich diet by upregulation of the alternative bile acid synthetic pathway may be an explanation for this effect (205,207). An interplay between other dietary factors and bile acids was shown *in vivo* in rats (304). Feeding rats a synthetic fat-free diet led to a decrease in bile acid synthetic capacity, possibly due to slower recirculation of bile acids along the enterohepatic axis in response to reduced functional need. In addition, Cheema *et al.* (305) showed in mice that the ability of dietary cholesterol to stimulate or repress the expression of cholesterol 7α-hydroxylase is dependent on the type of fatty acids in the diet.

A direct role of cholesterol in regulating bile acid synthesis was demonstrated using isolated rat hepatocytes. Bile acid synthesis can be stimulated by cholesterol-rich lipoproteins in cultured rat hepatocytes: ß-migrating very low density lipoprotein and intermediate density lipoprotein increased cholesterol 7α-hydroxylase activity, mRNA levels and transcriptional activity, while not affecting sterol 27-hydroxylase. The extent of stimulation of cholesterol 7α-hydroxylase is associated with the apolipoprotein E content of the lipoprotein particle. Transient transfection experiments in these cells revealed cholesterol-responsiveness within the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter (114, Post *et al.* unpublished data). In similar studies, transcriptional regulation by lipoprotein

cholesterol was observed in rat hepatoma and $HepG_2$ cells (111,306). However, regulatory elements were found more distally (111) Induction by cholesterol is probably regulated via as yet unidentified sequences within the promotor sequence. The motifs may differ from those within the promotor regions of HMG-CoA reductase and the LDL receptor, as cholesterol causes suppression of promoter activity of these genes (155), while exerting a positive effect on cholesterol 7α-hydroxylase, at least in a number of species. The presence of both sterol regulatory elements and bile acid-responsive elements may serve to titrate the expression of the cholesterol 7α-hydroxylase gene in response to cholesterol and bile acids returning to the liver via the EHC.

4.4.3 Oxysterols

Recently, it is suggested that it is not cholesterol but it oxidised metabolites which are responsible for stimulating cholesterol 7α-hydroxylase expression. Lehmann *et al.* (126) have identified a responsive element for the nuclear oxysterol receptor LXRα. In transfection experiments, this receptor was shown to be activated by a specific class of naturally occurring, oxysterols, including 22(*R*)-hydroxycholesterol, 24(*S*)-hydroxycholesterol, and 24,25(*S*)-epoxycholesterol (126,307). Mice lacking LXRα showed no induction of cholesterol 7α-hydroxylase after they were fed a cholesterol-rich diet (308), suggesting its important role in regulation by cholesterol. Next to the effect on LXR, oxysterols have been proposed to be activators of an other member of the nuclear receptor family, SF-1 (309,310). SF-1 can regulate the transcription of the steroidogenic acute regulatory protein (StAR), which plays a role in enhancing the delivery of cholesterol to the inner mitochondrial membrane in extrahepatic tissues (310). *In vitro* studies showed an increased activity of sterol 27-hydroxylase by StAR (311). Since the importance of this latter pathway has to be further investigated *in vivo*, these results suggest that oxysterols can have an effect via nuclear receptors on both the neutral as well as the acidic pathway.

In former studies, oxydation products of cholesterol, such as 7-oxocholesterol, have been also identified in response to cholesterol-feeding. Whereas several oxysterols, including 7-oxocholesterol, competitively inhibit binding of cholesterol to cholesterol 7α-hydroxylase (312), intravenous infusion or feeding of 7-oxocholesterol in the rat led to an unexpected up-regulation of cholesterol 7α-hydroxylase activity and mRNA (313,314). It was suggested that the competitive inhibitor 7-oxocholesterol provoked a decrease in bile acid synthesis, resulting in a

compensatory increase in levels of cholesterol 7α-hydroxylase mRNA and protein, as a consequence a decreased bile acid-induced feedback (313). In primary rat hepatocytes addition of four different oxysterols, among which 7α- and 27-hydroxycholesterol, failed to have a significant effect on expression of cholesterol 7α-hydroxylase mRNA (289). In addition, 25-hydroxycholesterol a potent regulator of HMG-CoA reductase and LDL receptor gene expression (153-155) had no effect on 7α-hydroxylase expression in $HepG_2$ cells (306). These latter oxysterols had relative little or no effect on activation of LXRα or LXRß (126,307). Futher studies are necessary to elucidate which oxysterol is involved in diet induced expression of cholesterol 7α-hydroxylase via the LXR pathway.

4.5 Drugs and other compounds affecting bile acid synthesis

Several drugs and other compounds have been described which can influence bile acid synthesis. The effects of a number of compounds were reviewed previously (25,75). More recent findings are described in short here. Some of the drugs studied are known to affect cytochrome P-450-dependent processes. The cytochrome P-450 inducer phenobarbital may have different effects on cholesterol 7α-hydroxylase activity in rats; no effect as well as stimulation was found (25,75). Various groups proposed that the effect of phenobarbital on the responsiveness of cholesterol 7α-hydroxylase in rats is related to strain differences (315,316). Among nine strains of rats, two showed increased levels of cholesterol 7α-hydroxylase activity and in mRNA 2-3 days after phenobarbital treatment, secondarily leading to upregulation of HMG-CoA reductase activity and mRNA in the liver (316). The mechanism of the induction is unknown. On the other hand, the hypolipidemic agent clofibrate, which also induces cytochrome P-450 failed to produce an increase in cholesterol 7α-hydroxylase in rats (25). *In vivo* data in humans showed a decrease in cholic acid synthesis (317,318) and the second generation fibric acid derivates, gemfibrozil and bezafibrate, were reported to suppress cholesterol 7α-hydroxylase activity and bile acid synthesis in man (319-321). Recently, we showed that ciprofibrate suppresses bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase via the peroxisome proliferator activator receptor (PPAR) α. The direct role of PPARα in this process was determined in PPARα -/- mice. Promoter-reporter studies demonstrated that a functional PPAR-responsive element is present in the proximal promoter of the rat cholesterol 7α-hydroxylase gene (Post *et al.* unpublished results). In the recently developed mouse with a null mutation in the

sterol carrier protein 2-/sterol carrier protein x-gene (Scp2) phytanic acid levels were increased, which can activate PPARα (322,323). Similar observations were made in the Scp2 -/- mice as compared to mice fed with fibrates i.e. peroxisome proliferation, hypolipidemia and enhanced expression of genes encoding peroxisomal ß-oxidation and a decreased expression of sterol 27-hydroxylase (322). However, the Scp2-/- showed increased expression of cholesterol 7α-hydroxylase in contrast to a decreased expression by fibrates, indicating a different mode of regulation for this enzyme for these PPARα activators.

Feeding rats a diet supplemented with the pregnenolone derivative pregnenolone 16α-carbonitrile (PCN) also resulted in down-regulation of cholesterol 7α-hydroxylase (85,88,324). The anti-mycotic drug ketoconazole, a lanosterol 14α-demethylase inhibitor and a potent inhibitor of cytochrome P-450-mediated processes strongly inhibited cholesterol 7α-hydroxylase activity, resulting in a decreased bile acid synthesis in cultured human and rat hepatocytes (325). The inhibition of bile acid synthesis by ketoconazole was confirmed *in vivo* in bile-diverted rats (325). In a later study, the same authors showed that this inhibition was followed by a marked overshoot of the process and of cholesterol 7α-hydroxylase activity in rats with an intact enterohepatic circulation (326). Another lanosterol 14α-demethylase inhibitor Azanlanstat (RS-21607) stimulated cholesterol 7α-hydroxylase activity in hamsters by a yet unknown mechanism (327). Additionally, other cytochrome P-450 inhibitors, like metyrapone (312,326) and SKF-525A (312) were also able to inhibit cholesterol 7α-hydroxylase activity in rat liver microsomes, but to a much lesser extent than ketoconazole. The oxysterol derivative 6-azacholest-4-en-3β-ol-7-one was found to be a noncompetitive inhibitor of cholesterol 7α-hydroxylase (328).

Princen and coworkers (70) showed the immunosuppressive drug cyclosporin A to decrease bile acid formation in cultured human and rat hepatocytes by inhibition of chenodeoxycholic acid synthesis, with sterol 27-hydroxylase, the first enzyme in the alternative pathway as the site of inhibition (70,71). Similar inhibition was found to occur in $HepG_2$ cells (329).

Recently, we showed that cafestol, the cholesterol-raising component in boiled coffee, potently inhibits cholesterol 7α-hydroxylase activity, mRNA and gene transcription in primary cultures of rat hepatocytes. Cafestol, a diterpenoid also suppresses sterol 27-hydroxylase but to a lesser extent. The suppression of bile acid synthesis probably leads to an increase in the intracellular regulatory pool of

cholesterol, resulting in a decrease in LDL-receptor mRNA levels (330). These effects were validated *in vivo* in apoE_3-Leiden transgenic mice (De Roos, Post *et al* unpublished results). In these mice, cafestol decreased bile acid synthesis, reflected by a reduction in the total amount of fecal bile acids excreted, by down-regulation of expression of enzymes involved in the neutral as well as in the alternative bile acid synthetic pathway. The consequent increase of hepatic cholesterol resulted in a decline in LDL-receptor mRNA levels and was removed from the liver by an increased secretion of VLDL cholesteryl esters. This may provide an explanation for the cholesterol-raising effect of unfiltered coffee in humans as found in a number of epidemiological and intervention trials.

5. LIVER HETEROGENEITY OF BILE ACID SYNTHESIS AND INTERACTION WITH CHOLESTEROL SYNTHESIS

The liver plays an important role in the homeostatic maintenance of a large number of nutrients in the blood, such as carbohydrates, amino acids and lipids, and is the main site of intermediary metabolism thereof. It has become increasingly clear that not all hepatocytes contribute equally in this task. In contrast, contribution of hepatocytes to uptake, storage, interconversion, and release of various compounds shows a large degree of heterogeneity along the portocentral axis (331,332).

It has been shown that cholesterol synthetic enzymes HMG-CoA reductase and HMG-CoA synthase are predominantly localized in the periportal hepatocytes, as judged from immunohistochemical staining (333,334). In contrast, bile acid synthesis and the key enzymes involved therein, cholesterol 7α-hydroxylase and sterol 27-hydroxylase, are localized mainly pericentrally, as demonstrated in collaborative studies by Princen and Gebhardt and coworkers (201,335). Preferential expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA and transcription was observed within the pericentral zone (201, Figure 7). The heterogenous expression of cholesterol 7α-hydroxylase develops during early life. Whereas the homogenous distribution is present in fetal rat livers, the lobular gradient appears at day 4 after birth resulting in an adult heterogenous expression pattern at day 28 (336). Thus, under normal conditions synthetic and catabolic routes of cholesterol are separated, limiting linkage of both pathways to a few cells within the acinus. In particular, these studies provide an explanation for the preferential utilization of preformed cholesterol for bile acid synthesis under these circumstances

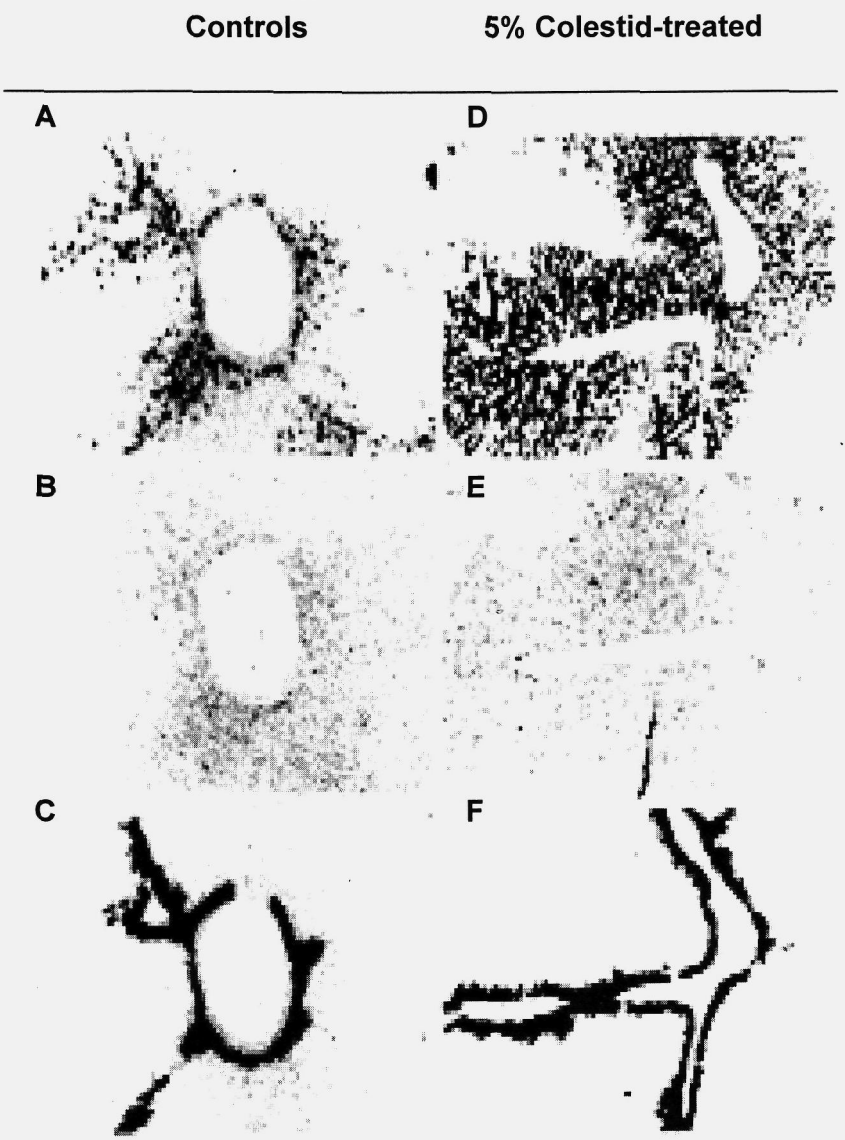


Fig. 7 Localization of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA on serial liver sections by in situ hybridization. Liver sections were made from control (a-c) and colestid-treated (d-f) rats and were hybridized in situ with ^{35}S-labeled probes for cholesterol 7α-hydroxylase (a and d), sterol 27-hydroxylase (b and e), and glutamine synthetase (c and f). The later was assessed as a positive identification of the pericentral zone. Sections depicted are of a central vein with surrounding hepatocytes. Reprinted with permission from Twisk J, Hoekman MFM, Mager WH, Moorman AFM, de Boer PAJ, Scheja L, Princen HMG, Gebhardt R. Heterogeneous expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes in the rat liver lobulus. J Clin Invest 1995; 95: 1235-1243.

(337). In addition, efficient biliary excretion of de novo synthesized cholesterol under normal circumstances (338) may be a consequence of the absence of bile acid synthetic enzymes in the portal region, where cholesterol is synthesized.

The distribution pattern of bile acid synthesis is not rigid, and the distribution patterns of the key-enzymes in bile acid synthesis can change in response to the amount of bile acids returning via the EHC. As reported, specific liver morphology results in the presence of a bile acid gradient over the liver acinus, with bile acid concentrations differing 6-fold between the periportal and pericentral extremes (339,340) and showing an inverse relationship with bile acid synthetic capacity (335). Conversely, administration of bile acid sequestrants is proposed to lower the acinar bile acid gradient (213,335), resulting in increased expression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase over a larger part of the liver acinus (201, Figure 7). The latter is in line with the concentration-dependent down-regulation of both enzymes, as demonstrated in cultured rat hepatocytes (97,133,196). Up-regulation of cholesterol catabolism in the portal region, as a consequence of interruption of the EHC, may in part be responsible for the reported increase in use of de novo synthesized cholesterol under those circumstances (24-26,337). Similarly, diurnal rhythm not only affects expression of cholesterol 7α-hydroxylase per se, but may also govern relative lobular levels. At 10 am, at the time of the lowest cholesterol 7α-hydroxylase activity, 7α-hydroxylase expression was limited to a few pericentral hepatocytes, whereas at 10 pm with the highest expression approximately one half of the hepatocytes contained cholesterol 7α-hydroxylase mRNA (240). The circadian variation in distribution pattern may be established by effectors which differ themselves in porto-central expression.

In conclusion, the liver can efficiently regulate formation of bile acids, in response to a particular demand, by displacing expression of key-enzymes to a larger or smaller area within the liver acinus, thereby either linking bile acid synthesis to cholesterol synthesis for sufficient supply of substrate or uncoupling the two routes by creating a spacial barrier between them. This mechanistic view may also put limits on the potential of bile acid sequestrants, as their use will draw on de novo synthesized cholesterol in addition to lipoprotein cholesterol. Therefore, combining sequestration of bile acids with the use of cholesterol synthesis inhibitors is a much more powerful alternative.

6 RECENT DEVELOPMENTS IN STIMULATION OF BILE ACID SYNTHESIS

6.1 Drugs affecting intestinal uptake of bile acids

Enhancement of bile acid synthetic capacity by interruption of the enterohepatic circulation of bile acids with the sequestrants cholestyramine or colestipol has been for a long time a way to decrease plasma cholesterol levels and the cardiovascular risk in hypercholesterolemic patients (9-15). These bile acid sequestrants are high-molecular weight cationic ion-exchange resins, which bind anionic bile acids and prevent their intestinal absorption. Major disadvantages, however, are their modest hypolipidemic effect and poor palatability. Patients must consume multi-gram doses (10-30g) to achieve a LDL cholesterol lowering of 10-20% (9-13). The sequestrants are quite inefficient in binding bile acids as only 5% of the available sites in the resin are occupied by bile acids. Since this type of drugs is not absorbed and may have, therefore, safety advantages over systemic agents, several attempts have been made to develop more efficacious compounds. Some former compounds are DEAE-Dextran and activated carbon beads. DEAE-Dextran is a water-soluble sequestrant, which is available in Italy. It is reported to be better tolerated on long-term administration, since smaller amounts are used. Cholesterol-lowering effects are modest, but the compound also reduces plasma triglycerides (341,342). Activated carbon beads were somewhat less active as cholestyramine in reducing plasma cholesterol (343).

To increase the potency of the compounds two approaches can be followed. Resins have been designed with increased binding capacity, e.g. by introducing a spacer arm to the polymeric backbone, or by making them water-soluble. Along the first line several companies have developed resins, which are claimed to be 2-3 fold more potent than cholestyramine, such as MCI-196 from Mitsubishi (344,345), SKF-97,426-A (346) and a number of other polymeric structures (e.g. 347) from SmithKline Beecham (see Figure 8), and a cross-linked polyallylamine containing N-octyl-N,N-dimethylammonium groups from ICI Pharmaceuticals (348). Water-insoluble bile acid sequestrants are also disclosed by Rohm and Haas claiming to have a three times greater efficacy than cholestyramine (349,350). Hoffmann-La Roche has patented a series of cationic phenyl-pyrimidinium, thiazolium or imidazolium salts claimed to inhibit intestinal cholesterol and bile acid absorption, but further data are lacking (351). A series of novel lipophilic polyamines has been patented by Upjohn, which were up to 29 times more potent than colestipol in lowering serum cholesterol levels in Japanese quail (352, Figure 8). Alpha-Beta

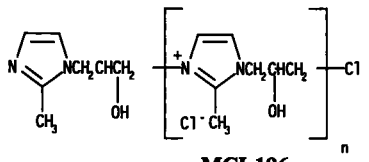
Technology patented a derivatized cationic polysaccaride, Cholazol H, an insoluble dietary fiber which combines the cholesterol-lowering properties of dietary fiber with the bile acid sequestrant properties of a hydrofobic catonized resin (353). This compound was shown to be as effective as cholestyramine for prevention of diet-induced hypercholesterolemia and early artherosclerosis in hamsters (354). Farmhispania (355) also patented derivatized cationic polysaccharides, which were claimed to bind bile acids and to lower plasma lipids.

In the second approach water-soluble agents were developed by incorporation of polyionic groups into the resin to increase efficacy such as the polyanhydroaspartic acid polymers from Berlex (356,357) and the Pfizer compound CP-88488, a chitosan derivative possessing a quaternary nitrogen containing side chain and being two times more potent than cholestyramine (358, Figure 8). DuPont-Merck (359,360) and GelTex (361,362) have patented polycationic polymeric hydrogels, which are claimed to be 6-fold and 10-fold, respectively, more potent than cholestyramine.

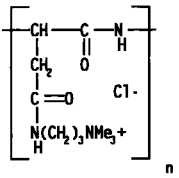
Still another approach is to interfere with the uptake of bile acids by developing compounds, which inhibit the intestinal bile acid transport system. This line has been followed by Beecham (363) and is pursued by Hoechst (364,365). Both companies have developed small compounds which mimic the action of a sequestrant without the use of bulky resins (Figure 8). In rabbits inhibition of the ileal Na^+/bile acid cotransporter by the inhibitor S-8921 from Shionogi Research Laboratories resulted in induction of cholesterol 7α-hydroxylase and decreased plasma cholesterol levels (366). A variety of more biotechnological approaches to increase bile acid synthesis has been proposed. The University of Texas has patented the use of a genomic DNA library for a cholesterol 7α-hydroxylase regulatory factor, which interacts with the 7α-hydroxylase promoter DNA, as a way to control cholesterol catabolism (367). Adenoviral transfection of the gene encoding cholesterol 7α-hydroxylase has recently been reported to increase bile acid synthesis and to favourably alter lipoprotein profiles in hamsters (19). However, further studies are neccessary to develope a gene-therapy for longer-term.

6.2 Dietary fibres and plant sterols interfering with uptake of bile acids

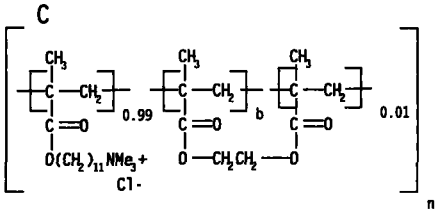
Intake of foods rich in soluble fiber has been shown to lower blood cholesterol levels by enhancing fecal loss of bile acids and cholesterol (368), for a recent review see Truswell (369). This finding has urged the food and drink industry to develop new



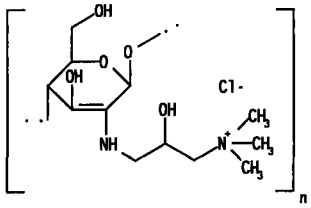
1 MCI-196 (Mitsubishi/Bristol-Myers Squibb)



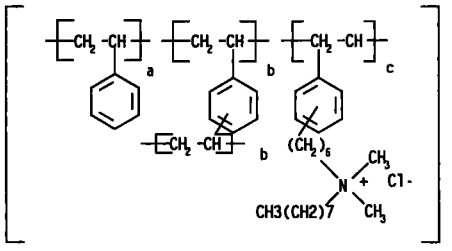
5 (Berlex)



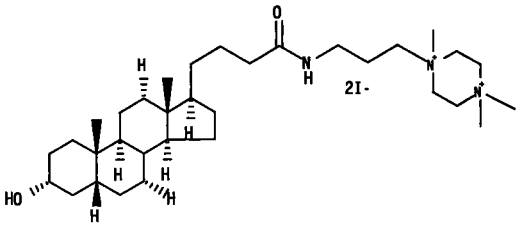
2 SKF-97,426-A (SmithKline Beecham)



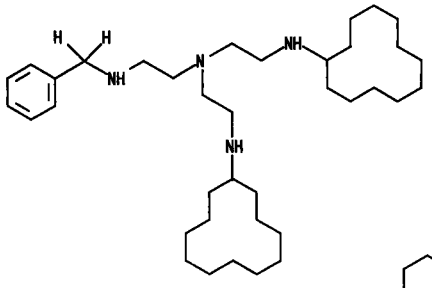
6 CP-88488 (Pfizer)



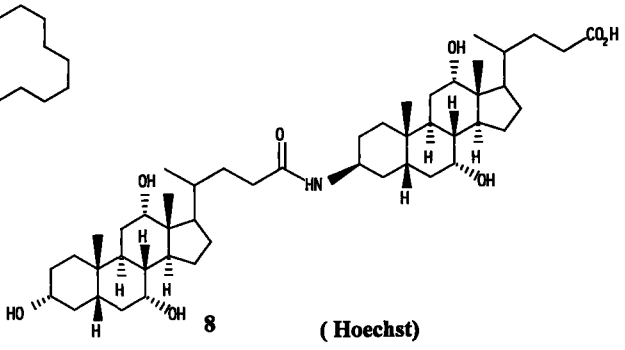
3 (SmithKline Beecham)



7 BRL 39,924A (SmithKline Beecham)



4 (Upjohn)



8 (Hoechst)

Fig. 8 Some bile acid sequestrants and other compounds inhibiting intestinal bile acid absorption.

"functional foods" or "nutraceuticals", which provide medical-health benefits, in this case by lowering plasma cholesterol levels. Sources of soluble dietary fiber are psyllium, guar gum, pectin and oat bran. In contrast, dietary fibres from insoluble, fibrous substances like cellulose and lignin do not lower cholesterol levels (369).

Psyllium, a mucilage from the seeds of Plantago ovata, has been reported to increase fecal excretion of bile acids in men (370,371) and to enhance cholesterol 7α-hydroxylase activity and mRNA in hamster and rat, which may explain its cholesterol-lowering effects (372-375). The fibre augmented the effect of cholestyramine (373). Guar gum is the most widely used dietary fibre in trials, since it lowers both plasma cholesterol and increased plasma glucose levels after meals in diabetics. The fibre, a complex carbohydrate, is used as a thickener in food processing and is a galactomannan from the seeds of the leguminous plant Cyamopsis tetragonobola. Guar gum increases fecal excretion of bile acids and cholesterol (369,376) and induces cholesterol 7α-hydroxylase (375). Further, it may repress lipogenesis, probably by the fact that the compound is fermented in the cecum (376). Pectins are complex carbohydrates usually isolated from citrus fruits, which lower cholesterol levels by sequestering bile acids in the intestine (369), causing upregulation of cholesterol 7α-hydroxylase (374,375). The active ingredient in oat bran is its gum, a β-glucan, which is responsible for intestinal binding of bile acids and it cholesterol-lowering activity (369,377,378). Several other amalyse-resistant complex carbohydrates, such as β-cyclodextrin and various forms of dietary-resistant starch, have been described, which also stimulate cholesterol 7α-hydroxylase activity (379), fecal excretion of bile acids and lower plasma cholesterol levels modestly (380-382).

In our institute the transgenic apoE_3-Leiden mice were generated, which are extremely sensitive for inhibitors of intestinal bile acid and cholesterol uptake. These mice have a disturbed clearance of apoE-containing lipoproteins and their plasma cholesterol and triglyceride levels depend, therefore, strongly on input of cholesterol into the circulation by chylomicron and VLDL production (383,384). We found strong decreases (approximately 50%) in plasma cholesterol levels upon feeding with cholestyramine and guar gum (unpublished data). The mice develop atherosclerosis upon cholesterol and fat feeding. Quantitative analysis has demonstrated that the lesion area is strongly correlated with serum cholesterol concentrations and exposure time (385). We propose that these mice are a good model to investigate

the potency of sequestrants and of dietary fibers to lower cholesterol levels and to study their effect on atherosclerosis.

Recent days, plant sterols (also called phytosterols) become more and more populair in the food industry as additive in margarine (386,387). Plant sterols can inhibit cholesterol absorption by displacing bile acid micelles. In some species it has been shown that plantsterols can be more potent than cholestyramine (388). Thus, plantsterol may increase bile acid synthesis by a reduced feedback inhibition of bile acids. However, plant sterols can also have opposite effects on bile acid synthesis. In patients with the genetic disorder phytosterolemia which is a rare lipid storage disease characterized by elevated concentrations of plant sterols in tissues (389), decreased bile acid synthesis can be found (390). This is due to competitive inhibition of hepatic cholesterol 7α-hydroxylase and sterol 27-hydroxylase by these plant sterols (391,392).

7. CONCLUDING REMARKS AND FUTURE DIRECTIONS

In the past few years our knowledge of regulation of cholesterol 7α-hydroxylase gene expression has rapidly expanded as a result of isolation and characterization of the gene and its regulatory sequences. Negative feedback regulation of bile acid synthesis by bile acids returning to the liver via the enterohepatic circulation is evidently the most important process which determines cholesterol 7α-hydroxylase activity. Future studies will be focused at the exact molecular mechanism by which expression of the cholesterol 7α-hydroxylase gene is modulated. Questions to be resolved are: Is there a bile acid receptor, acting in analogy with other nuclear transcription factors of the steroid-thyroid hormone superfamily? Or does modulation proceed via a signal transduction pathway involving second messengers? What is the contribution of post-transcriptional regulation, i.e. at the level of stabilization of the mRNA, and how does this take place? Is a post-translational mechanism active?

We have shown that, apart from hydrophobicity, structural elements in the bile acid molecule play an important role in regulation of cholesterol 7α-hydroxylase. Compartmentalization, in which the hydroxyl groups present on the bile acid are in close proximity and hence form a clear hydrophilic environment, may be a prerequisite for binding to a putative bile acid receptor involved in interaction with regulatory sequences or to other regulatory proteins. Apart from a mechanistical

point of view such knowledge can be important for the development of alternatives for bile acid sequestrants, which may involve the synthesis of compounds designed to interfere with interactions of bile acids with a negative regulator in bile acid synthesis (e.g. bile acid receptor antagonists).

New studies will be directed toward regulation of cholesterol 7α-hydroxylase by cholesterol and hormones and other bio-active regulatory molecules, and toward the molecular mechanism of their action. Much research has been performed in animal models and the development of different transgenic mice is of great value for the determination of contribution of the two pathways to bile acid synthesis. However, this phenomenon and/or regulation by certain factors may be different in humans. This emphasizes the need for validation in human systems, e.g. hepatocytes, and if possible in man himself. The identification of new inborn errors in bile acid synthesis in this point of view is very important.

The role of sterol 27-hydroxylase as well as the recently identified oxysterol 7α-hydroxylase as a site of regulation of bile acid synthesis needs further clarification. There is increasing evidence that sterol 27-hydroxylase is subject to certain regulatory factors e.g. bile acids and some hormones, *in vivo* and *in vitro*, but data are not consistent among species. Alternatively, the acidic pathway may act as a back-up route under conditions in which cholesterol 7α-hydroxylase activity is low or as a way to increase the diversity of bile acids. Therefore, further investigations are necessary to determine the contribution of oxysterol 7α-hydroxylase in the formation of bile acids and most important how this enzyme is regulated. The development of oxysterol 7α-hydroxylase knock-out mice would be useful for further studies. In addition, the various steps in the acidic pathway are not yet fully uncovered.

The opposite lobular localization of bile acid and cholesterol synthesizing enzymes is interesting, since it poses questions regarding the mechanism of interregulation of cholesterol and bile acid synthetic pathways and how different separated cholesterol pools may contribute to liver cholesterol homeostasis. The heterogeneous distribution indicates that a direct physical linkage between the cholesterol synthetic and catabolic pathways is at its best limited to only a few cells within the liver lobulus and that under normal circumstances the liver utilizes preformed cholesterol for bile acid synthesis.

It is tempting to speculate that the finding in animal studies that bile acid synthetic capacity is an important genetic factor, which may influence the

responsiveness to dietary cholesterol, may have its equivalent in humans. If so, genetic differences in bile acid synthetic capacity could explain in part different responses between humans to the effect of consumption of cholesterol-containing foods on plasma cholesterol levels. The interaction of cholesterol or its metabolite with a nuclear transcription factor and gene regulation leading to differences in bile acid synthesis has to be elucidate further. A good candidate in this process is LXR.

Promising developments have been reported by several pharmaceutical companies. A number of novel compounds have been designed, which are claimed to interrupt the EHC of bile acids and to decrease blood cholesterol levels more potently than cholestyramine and colestipol. These compounds may form a new generation of more palatable bile acid sequestrants and of other resorption-inhibiting drugs active in lowering plasma cholesterol levels. Furthermore, more insight in the molecular mechanism of regulation of different genes involved in bile acid synthesis could be of great value for drugs targeting and gene therapy.

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Chapter 2

Lipoprotein cholesterol uptake mediates upregulation of bile acid synthesis by increasing cholesterol 7α-hydroxylase but not sterol 27-hydroxylase gene expression in cultured rat hepatocytes.

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Lipoprotein cholesterol uptake mediates upregulation of bile acid synthesis by increasing cholesterol 7α-hydroxylase but not sterol 27-hydroxylase gene expression in cultured rat hepatocytes

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Running title: Upregulation of cholesterol 7α-hydroxylase by cholesterol-rich lipoproteins

ABSTRACT

Lipoproteins may supply substrate for the formation of bile acids, and the amount of hepatic cholesterol can regulate bile acid synthesis and increase cholesterol 7α-hydroxylase expression. However, the effect of lipoprotein cholesterol on sterol 27-hydroxylase expression and the role of different lipoproteins in regulating both enzymes are not well established. We studied the effect of different rabbit lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cultured rat hepatocytes. ß-migrating very-low-density lipoprotein (ßVLDL) and intermediate-density-lipoprotein (IDL) caused a significant increase in the intracellular cholesteryl ester content of cells (2.3- and 2-fold, respectively) at a concentration of 200 µg cholesterol/ml, while high-density-lipoprotein (HDL) (50 % v/v), containing no apo E, showed no effect, after a 24-hour incubation. ßVLDL and IDL increased bile acid synthesis (1.9- and 1.6-fold) by upregulation of cholesterol 7α-hydroxylase activity (1.7- and 1.5-fold, respectively). Dose- and time-dependent changes in cholesterol 7α-hydroxylase mRNA levels and gene expression underlie the increase in enzyme activity. Incubation of cells with HDL showed no effect. Sterol 27-hydroxylase gene expression was not affected by any of the lipoproteins added. Transient expression experiments in hepatocytes, transfected with a promoter-reporter construct containing the proximal 348 nucleotides of the rat cholesterol 7α-hydroxylase

promoter, showed an enhanced gene transcription (2-fold) with ßVLDL, indicating that a sequence important for a cholesterol-induced transcriptional response is located in this part of the cholesterol 7α-hydroxylase gene. The extent of stimulation of cholesterol 7α-hydroxylase is associated with the apo E content of the lipoprotein particle, which is important in the uptake of lipoprotein cholesterol.

We conclude that physiological concentrations of cholesterol in apo E-containing lipoproteins increase bile acid synthesis by stimulating cholesterol 7α-hydroxylase gene transcription, whereas HDL has no effect and sterol 27-hydroxylase is not affected.

INTRODUCTION

Most species respond to an increase in the dietary load of cholesterol by suppressing endogenous cholesterol synthesis and by utilising the hepatic capacity to store large quantities of cholesterol in its esterified form. Several groups have demonstrated that feeding a high-cholesterol diet also results in up-regulation of bile acid synthesis (For review see 1) illustrating the importance of this metabolic pathway for maintaining cholesterol homeostasis. Bile acid synthesis and biliary secretion of cholesterol into the bile is the only quantitative way of eliminating cholesterol from the body (2,3).

The primary route to bile acids is initiated by 7α-hydroxylation of cholesterol by cholesterol 7α-hydroxylase, a microsomal cytochrome P450-dependent enzyme (4-6). However, there are also alternative pathways to bile acid synthesis operational (1,7,8). One of these pathways is initiated by 27-hydroxylation of cholesterol by sterol 27-hydroxylase, a cytochrome P450-dependent enzyme, located in the inner mitochondrial membrane (9-13). This alternative pathway can contribute substantially to bile acid synthesis in cultured human and rat hepatocytes (12,13) and *in vivo* in humans (7), rats (14) and rabbits (15). Both enzymes are regulated co-ordinately by a number of mediators (13,16-19).

A regulatory role has also been ascribed to cholesterol, although the mechanism of regulation is controversial. A mode of regulating bile acid biosynthesis by cholesterol has been suggested which involves the saturation of the enzyme cholesterol 7α-hydroxylase (20-22). However, Einarsson *et al.* (23,24) did not find such a relationship. Several groups have shown that feeding rats a cholesterol-rich

diet led to an increased cholesterol 7α-hydroxylase activity, mRNA level, and transcriptional activity (25-30). It remains unclear, however, whether cholesterol exerts its effect directly on cholesterol 7α-hydroxylase or whether other factors may also contribute. Constant infusion of mevalonate, a cholesterol precursor, in control and bile-fistulated rats stimulated cholesterol 7α-hydroxylase gene expression, suggesting a direct involvement of cholesterol (31,32). An indirect mechanism was also proposed, in which stimulation of cholesterol 7α-hydroxylase by dietary cholesterol is ascribed to malabsorption of bile acids in the intestine, resulting in a reduced potential for bile acid-induced feedback or to the involvement of an intestinal factor (28,33-35)

Furthermore, the effect of lipoprotein cholesterol on sterol 27-hydroxylase expression and the role of different lipoproteins in regulating both enzymes are not well established. In the current study, we have re-assessed the role of lipoprotein cholesterol as a regulator of bile acid biosynthesis (21,36-39) in cultured rat hepatocytes which has the advantage of allowing us to discriminate between direct and indirect events. The effects of different lipoproteins as a source of exogenous cholesterol were assessed on the regulation of the two key enzymes of this metabolic route. Our results show that cholesterol or a metabolite thereof supplied by cholesterol- and apo E-rich lipoproteins stimulates bile acid synthesis by directly stimulating cholesterol 7α-hydroxylase gene expression, while sterol 27-hydroxylase remains unaffected.

MATERIAL AND METHODS

Materials and animals

Materials used for the isolation and culturing of rat hepatocytes, and for assaying cholesterol 7α-hydroxylase were obtained from sources described previously (19,40,41). [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]dUTP (400 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK. Cholesterol kit (catalogue no.125512) and triglyceride kit (catalogue no. 701904) were provided by Boehringer, Mannheim, Germany. The phospholipid kit (#990-54009) was provided by Wako Chemicals, Neuss, Germany. CI-1011 was kindly provided by Dr. Krause, Parke Davis Pharmaceutical Research Division, Ann-Arbor, MI, USA.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water *ad libitum*. In the experiments described rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands) two days before the isolation of hepatocytes. For the preparation of

hepatocytes, the animals were killed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously (19,40,41). After a 4-hour attachment period, the cell medium was refreshed with 1.0 ml (6-well plates for: intracellular lipids (1 well) and transfection experiment (3 wells)) or 2.5 ml (60 mm diameter dishes for: bile acid mass production (2 dishes), enzyme activity (5 dishes), mRNA (1 dish) and nuclear run-off assay (7 dishes)) of Williams E (WE) medium supplemented with 10% (v/v) FCS, and cells were incubated for a further 14 hours. Lipoproteins in medium containing 10% (v/v) lipoprotein-deficient serum (LPDS) were added to the cells after this period, between hour 18 and hour 42 of culture age, unless otherwise stated. Cells were harvested at the same time after a 42-hour culture period for measurement of intracellular lipids, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, mRNA levels, and the determination of transcriptional activity.

Isolation of LPDS; lipoprotein isolation and characterisation

LPDS was isolated from FCS (Boehringer Mannheim) by ultracentrifugation at 4°C for 48 h after a density adjustment with solid KBr. The LPDS fraction (d> 1.21 g/ml) was dialysed at 4°C against 10 mM sodium phosphate, 0.15 M NaCl (pH 7.4) for 24 h, and subsequently against WE medium for an additional 24 h, and sterilised by filtering through a 0.22 µm membrane.

For the isolation of lipoproteins, blood was obtained from rabbits fed a diet supplemented with 1% (w/w) cholesterol for 7 days. Rabbit lipoproteins were chosen based on the fact that only one rabbit (without sacrifice) is needed to obtain sufficient amounts of lipoproteins in contrast to the large number of rats needed for isolation of rat lipoproteins. In addition, sufficient amounts of ß-migrating very-low-density lipoprotein (ßVLDL)-like particles in rat can only be obtained by feeding cholate-containing diets rich in fat and cholesterol having the disadvantage of contamination of lipoproteins with cholate even after dialysis. Lipoproteins were isolated by ultracentrifugation (40000 revs./min) in a SW40 rotor (18 hr, 4oC) on a NaCl density gradient: 4 ml serum (d (ρ) of 1.21 withKBr) + 2.6 ml NaCl (ρ1.063) + 8.6 ml H2O. Gradients were fractionated and cholesterol and triglyceride levels in lipoprotein fractions were determined enzymatically, using commercial kits. The different lipoprotein fractions were pooled on the basis of the determination of density (density measuring cell DMA 602M Mettler/Paar, Graz, Austria) and cholesterol profile (see Fig. 1a). The ßVLDL-fraction (d< 1.006) contained most of the lipoprotein-associated serum cholesterol (12.3 mg/mg protein), was rich in phospholipids (2.16 mg/mg protein), and contained relatively low amounts of triglycerides (0.22 mg/mg protein), as a consequence of displacement by cholesterol, giving this particle a ß-migrating mobility. IDL (d=1.004-1.030) contained less cholesterol (3.79 mg/mg protein), phospholipid (1.11 mg/mg protein) and triglycerides (0.03 mg/mg protein). The HDL-fraction (d=1.081-1.146) contained low amounts of cholesterol (0.57 mg/mg protein) and phospholipids (0.42 mg/mg protein), and hardly any triglycerides (0.011 mg/mg protein). Lipoprotein fractions were further analysed by SDS-polyacrylamide gel electrophoresis (Fig. 1b) with 4 to 20% gradient gels. ßVLDL predominantly contained apo E, with minor amounts of apo B100 and apo B48. IDL contained apo E and more apo B100 and apo B48 than ßVLDL. There was no contamination with HDL, since apo AI was

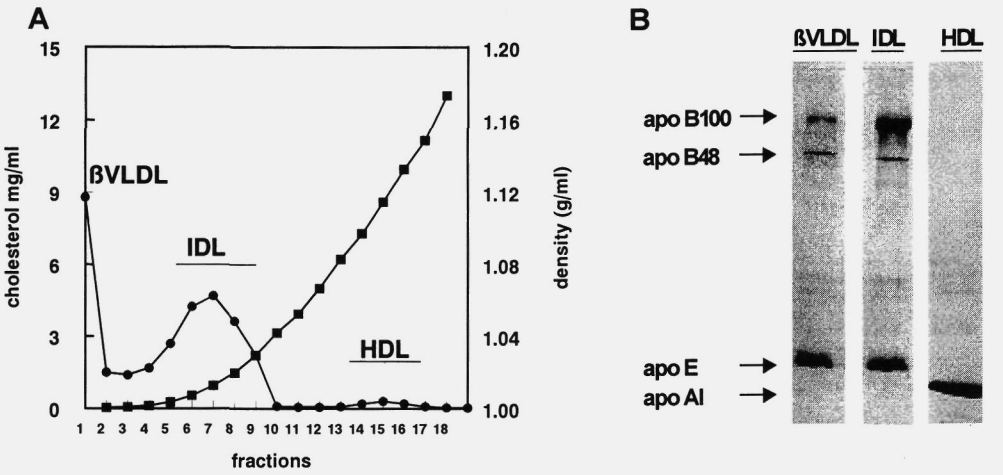

Fig. 1 Lipoprotein profile.
Serum from rabbits, fed a 1% cholesterol (w/w) diet for 1 week was fractionated by ultracentrifugation on a NaCL density gradient (squares). The cholesterol content (circles) of each individual fraction was measured enzymatically. Fraction 1 represents ßVLDL, fraction 5-9 IDL and fraction 13-17 HDL (Fig. 1a). Lipoprotein fractions were further analysed by SDS-polyacrylamide gel electrophoresis (Fig. 1b) with 4 to 20% gradient gels.

absent. HDL was rich in apo AI and contained no apo E. The ratio of apo E/apo B was determined by scanning the SDS-polyacrylamide gels with a Hewlett Packard Scanjet 4c and quantifying the
bands by image analysis using the program Tina version 2.09. The various fractions were stabilised by the addition of 10% (v/v) heat-inactivated LPDS (to prevent aggregation and oxidation), dialysed for 24 h against sodium phosphate buffer, for another 24 h against WE medium and then filtered through a 0.45 µm membrane. The fractions were stored at 4°C and used within 3 days.

Measurement of the mass of intracellular lipids
After a 24-hour incubation period, with or without lipoproteins, cells were washed 3 times with cold phosphate-buffered saline (pH 7.4) and harvested by scraping. Scraped cells were homogenised, and samples were taken for measuring the protein content and lipids were extracted from the cell suspension, after the addition of cholesterol acetate (2 µg per sample) as an internal standard. The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 precoated plates as described (19,42). Quantification of the amounts was done by scanning the plates with a Hewlett Packard Scanjet 4c and image analysis using the program Tina version 2.09.

Quantification of mass production of bile acids
Mass production of bile acids by rat hepatocytes was measured by gas-liquid-chromatography. Rat hepatocytes were cultured for 24 hours (from 26-50 hours of culture age) after a preincubation period of 8 hours (from 18-26 hours of culture age) in WE medium

containing 10% LPDS in both periods in the absence (control) or presence of different lipoproteins as described previously (19).

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity
Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were measured by HPLC according to (43) with minor modifications measuring mass conversion of cholesterol into 7α-hydroxy and 27-hydroxycholesterol. In short, 4 mg of protein of cell homogenates was incubated in 1 ml of buffer containing 0.1 M potassium phosphate buffer (pH 7.2), 50 mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v) and 0.015 % (w/w) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS). Twenty µl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated under agitation for 10 min at 37 °C. Then 200 µl of a regenerating system was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min 60 µl of a stop solution containing 20% (w/v) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. Steroid products were oxidised at 37°C for 45 min after the addition of 100 µl buffer containing 0.1% cholesteroloxidase (Calbiochem, USA catalogue no. 228250) (w/v), 10 mM potassium phosphate pH 7.4, 1 mM DTT and 20% glycerine (w/w), and the reaction was stopped by 2 ml ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether. The ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analysed by using C-18 reverse phase HPLC using a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitrile and 30% methanol at a flow rate of 0.8 ml/min. The amount of product formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, Cambridge, U.K.).

RNA isolation, blotting and hybridization procedures
Isolation of total RNA and subsequent electrophoresis, Northern-blotting and hybridisation techniques were performed as described previously (19). The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesised fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (41); a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, a 1.2 kb PstI fragment of hamster ß-actin cDNA, and a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used (19). The actin or GAPDH mRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantified using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Nuclear Run-Off Studies
Nuclear run-off studies were conducted essentially as described in ref. 41.
Hybridization - Target DNA, being 5 µg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster actin, rat GAPDH (see above) and the empty vector pUC19, were slot-blotted onto strips of Hybond-N+ filter (Amersham), and crosslinked with 0.4 M NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer, and hybridized with the labelled RNA for 36 hours. Labelled

RNA generated by incorporation of [^{32}P]-dUTP had been incorporated into nascent RNA, using isolated nuclei from cells which had been cultured with or without ßVLDL for a different lengths of time between 24 and 48 hours of culture time. Afterhybridization, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC (where 1xSSC is 0.15 M NaCl/0.015M sodium citrate)/1% SDS at 65°C, and exposed for 2-5 days to Phosphor Imager plate. Quantification of relative amounts of transcribed mRNA was performed using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Transfection experiments and CAT assays:
At 22 hours after isolation, cells were subjected to transfection using plasmid -348Rcat, subsequently cultured in medium (WE) containing 10% LPDS with or without ßVLDL (400µg cholesterol/ml) for 48 hours, and CAT-assays were performed, as described previously (44). -348Rcat contains the proximal 348 nucleotides of the rat cholesterol 7α-hydroxylase promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, used as a reporter. The amounts of acetylated product as represented by autoradiography were quantified by Phosphor-imager analysis. Data were corrected for protein-content and transfection efficiency.

Statistical analysis
Data were analysed statistically using Student's paired t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± S.E.M. Spearman's correlation coefficients were calculated to determine the association between apo E/apo B ratio and the induction of cholesterol 7α-hydroxylase mRNA or cholesteryl ester content in the cell.

RESULTS

Effect of different lipoproteins on intracellular lipid levels

To address the effects of an exogenous cholesterol source on the bile acid synthetic capacity of a liver cell, rat hepatocytes were incubated with rabbit lipoproteins of varying buoyant density, and cellular lipid content was determined as a measure of their internalisation (Table 1). Incubation with ßVLDL or IDL (both 200 µg cholesterol/ml) for 24 hours resulted in a 2.3- and 2-fold increase in intracellular cholesteryl ester content, respectively. HDL (50% v/v, which is equal to approximately 60 µg cholesterol/ml) did not change the cholesteryl ester content. Under the various incubation conditions intracellular free cholesterol and triglycerides levels were not significantly affected. The increase in cholesteryl ester content by ßVLDL was prevented by the simultaneous addition of the Acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitor CI-1011. A similar result was obtained when IDL was added together with CI-1011 (data not shown).

Table 1. Effect of different lipoproteins on intracellular lipid levels and bile acid mass production

Cholesterol source	Intracellular lipid levels			Bile acid synthesis
	CE	FC	TG	
	µg/mg cell protein			(% of control)
Control	10.6 ± 1.5	6.1 ± 1.2	10.5 ± 2.2	100
ßVLDL	24.8 ± 1.6***	6.5 ± 0.4	10.3 ± 0.2	191 ± 14*
IDL	21.2 ± 1.5**	6.4 ± 0.2	9.9 ± 0.6	164 ± 17*
HDL	11.9 ± 0.6	6.1 ± 0.2	11.7 ± 2.0	101 ± 20
ßVLDL + CI-1011	11.2 ± 0.6	7.1 ± 0.4	11.2 ± 0.8	N.D.

For measurement of intracellular lipids hepatocytes were incubated for 24 hours, from 18 to 42 h of culture, in the presence of lipoproteins (ßVLDL and IDL (200 µg cholesterol/ml), or 50% (v/v) HDL (containing approximately 60 µg cholesterol/ml)). Additional incubations with ßVLDL contained 3 µM of the ACAT inhibitor CI-1011. Lipoproteins used were isolated from rabbits fed a 1% cholesterol-enriched diet as described in the experimental section. Cells were harvested and the cellular content of cholesteryl ester (CE), free cholesterol (FC), triglycerides (TG) was determined as described. Values shown are expressed as mean absolute amounts (µg/mg cellular protein) ± S.E.M. For measurement of bile acid synthesis, rat hepatocytes were cultured for 24 hours (between 26-50 h of culture age) after an 8-hour preincubation period (from 18-26 h of culture age), in both periods in the absence (control medium containing 10% LPDS) or in the presence of the different lipoproteins. Bile acid synthesis is expressed as a percentage of the synthesis in control incubations and values shown are means ± S.E.M. The absolute bile acid synthesis rate in controls (10% LPDS only) was 3.68 ± 0.86 µg bile acids/mg cellular protein/ 24 hours. All values shown are obtained from independent experiments with hepatocytes from 3-7 rats. A significant difference between control and treated cells is indicated by asterisks (*P<0.05,** P<0.005, *** P<0.0005). (N.D.; not determined).

Effect of different lipoproteins on bile acid mass production

Bile acid mass production was measured over a 24-hour incubation period after a preincubation of 8 hours with the various lipoproteins as described in "Material and Methods". Incubation of hepatocytes with ßVLDL or IDL resulted in a 1.9- and 1.7-fold increase in bile acid mass production, respectively (Table 1). The major bile acids formed were cholic acid and ß-muricholic acid, in a ratio of approximately 20:80. This ratio did not change upon incubation with the various lipoproteins.

Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels.

To assess the level at which exogenous cholesterol, supplied by its physiological carriers, enhances bile acid mass production, enzyme activities and mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were determined. Rat hepatocytes were cultured for 24 hours in presence of ßVLDL, IDL or HDL, since cholesterol 7α-hydroxylase mRNA levels were maximally increased after a 18-24 hour incubation period (Fig. 2). Addition of ßVLDL or IDL stimulated cholesterol 7α-hydroxylase activity 1.7- and 1.5-fold, respectively. The increase of cholesterol 7α-hydroxylase activity was similar to the induction of its mRNA, being 1.8-fold with ßVLDL and 1.7-fold with IDL (Table 2). The induction of cholesterol 7α-hydroxylase mRNA levels was time- (Fig. 2) and dose-dependent (Fig. 3). Upon incubation of hepatocytes with ßVLDL, a maximal induction of cholesterol 7α-hydroxylase mRNA was observed with an equivalent of 400 µg cholesterol/ml. HDL did not increase cholesterol 7α-hydroxylase activity and mRNA levels. In contrast, sterol 27-hydroxylase activity was not affected by any of the lipoprotein fractions tested, indicating that the two enzymes diverge with respect to regulation by substrate cholesterol.

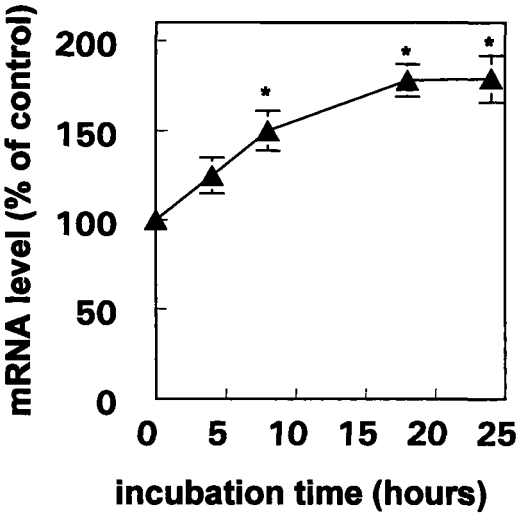

Fig. 2 Time course of stimulation of Cholesterol 7α-hydroxylase mRNA by ßVLDL

Rat hepatocytes were incubated with ßVLDL (200 µg cholesterol/ml) for different lengths of time, between 18-42 hours of culture, and were harvested simultaneously at 42 hours of culture time. Total RNA was isolated and cholesterol 7α-hydroxylase mRNA levels were determined relative to GAPDH mRNA expression as described in the experimental section. Data are expressed as a percentage of mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 3 rats. A significant difference (*P<0.05) between control and treated cells is indicated by an asterisk.

Table 2. Effect of different lipoproteins on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels

source of cholesterol	cholesterol 7α-hydroxylase		sterol 27-hydroxylase	
	activity	mRNA	activity	mRNA
Control	100	100	100	100
ßVLDL	170 ± 21*	179 ± 13**	91 ± 18	97 ± 5
IDL	151 ± 10*	165 ± 25*	102 ± 13	96 ± 8
HDL	61 ± 16	107 ± 14	82 ± 3	97 ± 4

Rat hepatocytes were incubated for 24 hours, from 18 to 42 h of culture, without (10% LPDS) or with ßVLDL, IDL (both at a concentration of 200 µg cholesterol/ml), or 50% (v/v) HDL (containing approximately 60 µg cholesterol/ml). Cells were harvested after 24 hours of incubation to measure cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activities and mRNA levels. Values shown are expressed as a percentage of enzyme activity or mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 3 to 6 rats. The absolute values for enzyme activity in controls cultured in 10% LPDS only were 352 ± 38, and 79 ± 18 pmol/mg cellular protein per hour, respectively, for cholesterol 7α-hydroxylase and sterol 27-hydroxylase. A significance difference between control and treated cells is indicated by an asterisk (*P<0.05, ** P<0.005).

As indicated above intracellular free cholesterol levels remained unchanged. Small changes in free cholesterol are difficult to detect due to the high amount of free cholesterol in the cell membranes, which may overshadow subtle changes in free cholesterol, and the rapid esterification of free cholesterol. To postulate a role for the regulatory pool of cholesterol in the regulation of cholesterol 7α-hydroxylase we measured mRNA levels of the LDL-receptor and 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase as sensitive measures to detect subtle changes in this pool. The latter mRNA levels decreased significantly (P<0.05) upon incubation with ßVLDL, by -33 ± 11% and -49 ± 7%, respectively (n=4). These data point to the involvement of cholesterol or a metabolite as a regulatory factor. mRNA levels of ß-actin and GAPDH, used as internal standards, were not affected by the lipoproteins. Simultaneous addition of both ßVLDL (200 µg cholesterol/ml) and CI-1011 (3 µM), to further increase the amount of regulatory cholesterol, resulted in even higher cholesterol 7α-hydroxylase mRNA levels as compared to incubations with ßVLDL alone (286 ± 21% versus 179 ± 13% (n=5; P<0.05)), further indicating that cholesterol or a metabolite is the regulatory compound. Even under these conditions

with an enhanced intracellular regulatory free cholesterol content, sterol 27-hydroxylase remained unaffected.

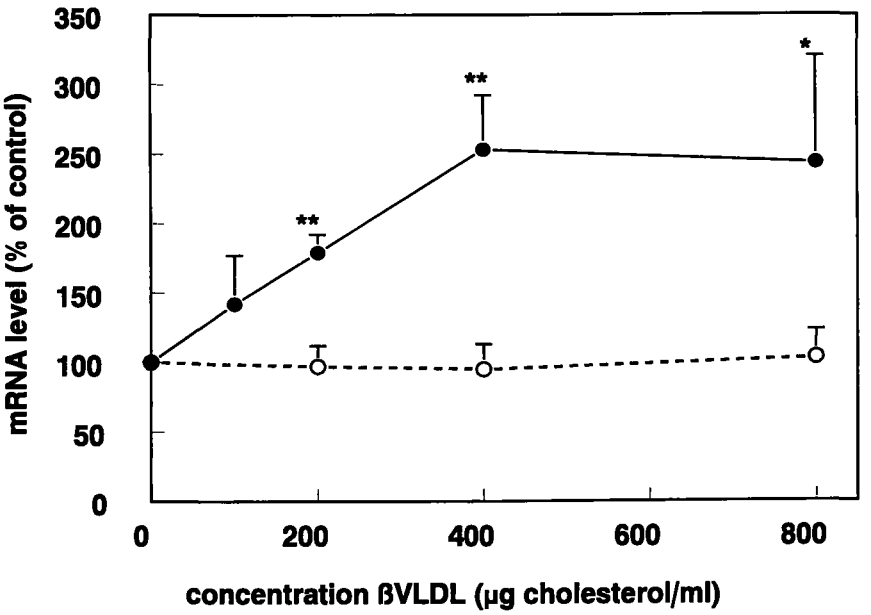

Fig 3. Dose-dependency of stimulation of cholesterol 7α-hydroxylase mRNA by ßVLDL
Rat hepatocytes were incubated for 24 hours, from hour 18 to hour 42 of culture, with various amounts of ßVLDL (100-800 µg cholesterol/ml). Total RNA was isolated and cholesterol 7α-hydroxylase (closed symbols) and sterol 27-hydroxylase (open symbols) mRNA amounts were determined, relative to GAPDH mRNA expression, as described in the experimental section. Data are expressed as a percentage of mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 3-6 rats. A significant difference (*P<0.05, ** P<0.005) between control and treated cells is indicated by an asterisk.

Effect of ßVLDL on the transcriptional activity of the cholesterol 7α-hydroxylase and sterol 27-hydroxylase genes.

To further examine the molecular level, at which the effect of lipoproteins is exerted, the transcriptional activity of the cholesterol 7α-hydroxylase gene was determined by means of nuclear run-off assays. Hepatocytes were incubated with ßVLDL for up to 24 hours, and harvested for the isolation of nuclei. Cholesterol 7α-hydroxylase transcriptional activity was rapidly induced upon incubation of cells with ßVLDL (200 µg cholesterol/ml): gene expression was significantly higher (1.4-fold) after 1.5 h of incubation, and maximally stimulated upon 3-12 h of ßVLDL treatment (1.9-fold) (Fig. 4).

The transcriptional activity of the sterol 27-hydroxylase gene was not affected by ßVLDL, in accordance with the absence of effects on sterol 27-hydroxylase activity and mRNA expression. The transcriptional activities of the ß-actin and GAPDH genes, used as internal standards, showed no changes upon the addition of ßVLDL.

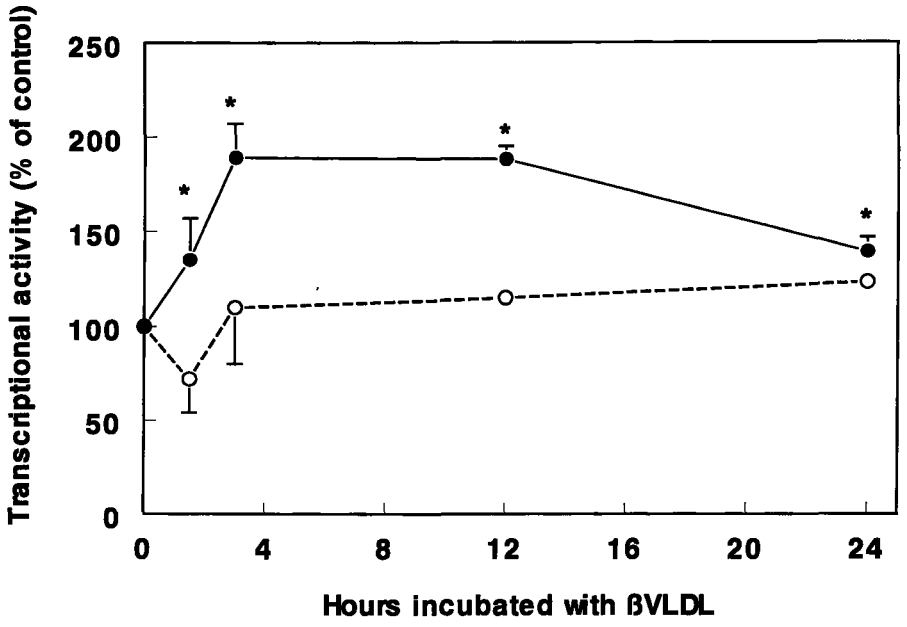

Fig 4. Time course of cholesterol 7α-hydroxylase and sterol 27-hydroxylase transcriptional activity in response to ßVLDL

Rat hepatocytes were incubated with ßVLDL (200 µg cholesterol/ml) for different lengths of time between hour 18 and hour 42 of culturing, and hepatocytes were harvested simultaneously after 42 h of culture time for the preparation of nuclei. Transcriptional activity of the different genes was determined by nuclear run-off assay, as described in "Experimental", and values are presented relative to transcriptional activity of the ß-actin gene, used as an internal standard. Non-specific hybridisation was checked using the empty vector pUC19. Relative transcriptional activities of cholesterol 7α-hydroxylase (closed) and sterol 27-hydroxylase (open) are expressed as a percentage of control value and are means ± S.E.M. of independent experiments with hepatocytes from 3-4 rats. A significant difference (*P<0.05) between control and treated cells is indicated by an asterisk.

Effect of ßVLDL on cholesterol 7α-hydroxylase promoter activity in cells.

Hoekman et al. (44), and others (45,46) have described a region in the proximal promoter of the rat cholesterol 7α-hydroxylase gene, harbouring major transcription-

regulating elements, responsive to a variety of physiological signals. To assess whether this particular region of the cholesterol 7α-hydroxylase promoter is also responsive to lipoprotein-cholesterol, transient expression studies were performed. The -348Rcat-construct, consisting of the first 348 base pairs of the rat cholesterol 7α-hydroxylase promoter fused to the CAT-reporter gene (44) was used in these experiments. Promoter activity of the construct was significantly ($P<0.05$) stimulated in the presence of ßVLDL (400 µg cholesterol/ml) as compared to control incubations (195% ± 7% versus 100% ± 10%). The data are expressed as a percentage of control and are means ± S.E.M. of independent experiments with hepatocytes from 4 rats. The data are in accordance with the approximately 2-fold stimulation of the transcriptional activity of the cholesterol 7α-hydroxylase gene as determined by nuclear run-off assays.

Relationship between cellular CE content or cholesterol 7α-hydroxylase mRNA levels and apo E/apo B ratio of lipoproteins.

The Apo E content of a particle determines in part the efficiency of uptake by a liver cell (47). Hence, apo E-rich lipoproteins are cleared more rapidly than those that rely solely on apo B-mediated uptake by the LDL-receptor.

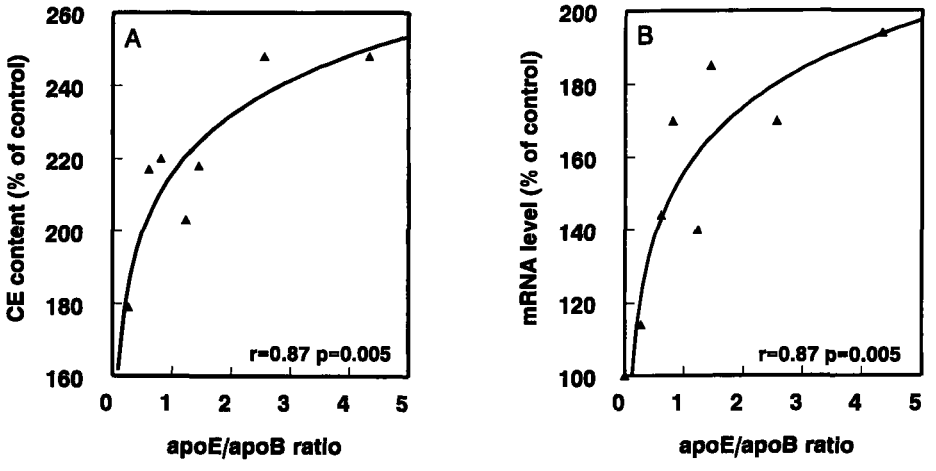

Fig 5. Relationship between cellular CE content or cholesterol 7α-hydroxylase mRNA levels and apo E/apo B ratio of lipoproteins.
Rat hepatocytes were incubated with different ßVLDL and IDL fractions (all at a concentration of 200 µg cholesterol/ml) from hour 26 to hour 42 of culture. Cells were harvested at 42 hours to measure the intracellular cholesteryl ester content (CE) and mRNA levels. The ratio of apo E/apo B was determined as described in the experimental section. Spearman's correlation coefficients were calculated to determine the association.

We therefore determined whether there was a relationship between apo E content of a particle, and its capacity to elevate intracellular cholesteryl ester levels. Subsequently, we also determined whether apo E content correlated with ability to induce cholesterol 7α-hydroxylase mRNA levels.

Cells were incubated with different ßVLDL and IDL subfractions, with different apo E/apo B ratio (fraction 1, 2-4, 5-7, 8-9) (Fig. 1a)). Equal amounts of cholesterol were added in each case. There was a positive correlation between the apo E/apo B ratio of the particle, and the extent of increase in intracellular cholesteryl ester content, (Fig. 5a) indicating a more efficient uptake of apo E-rich lipoproteins. As a consequence of the greater elevation in intracellular cholesteryl ester content, the higher apo E/ apo B ratio also resulted in elevated cholesterol 7α-hydroxylase mRNA levels (Fig. 5b).

DISCUSSION

This study shows that in rat hepatocytes, exogenous cholesterol supply in the form of lipoproteins stimulates the rate-limiting enzyme in the neutral pathway to the formation of bile acids, cholesterol 7α-hydroxylase. ßVLDL and IDL, both cholesterol-rich lipoproteins, induced cholesterol 7α-hydroxylase at the level of gene transcription, whereas the expression of sterol 27-hydroxylase was not affected. Elevation of the initial level of bile acid synthesis by feeding the rats with chow supplemented with 2% cholestyramine prior to isolation of the hepatocytes (13,19,41) was not found to be obligatory to observe upregulation of cholesterol 7α-hydroxylase by lipoprotein cholesterol.

We demonstrated that lipoprotein cholesterol has a direct stimulatory effect on cholesterol 7α-hydroxylase gene expression, in addition to having a merely stimulatory effect on bile acid synthesis by supplying cholesterol (21,36-39), and that not all lipoprotein fractions contribute to the stimulation. ßVLDL and IDL were active inducers, whereas HDL did not have an effect. The effect was accompanied by the accumulation of cholesteryl esters in the hepatocytes, as also observed *in vivo* (29), indicating that uptake of the lipoprotein cholesterol is important for regulation. This contention is supported by the strong association between the apo E content of the lipoprotein particle, expressed as the apo E/apo B ratio, and the extent of stimulation of cholesterol 7α-hydroxylase. The role of apo E in receptor-mediated uptake of

lipoproteins is well known (47). The HDL-fraction did not have an effect on cholesterol 7α-hydroxylase expression, due to the fact that this fraction did not contain apo E and contained significantly less cholesterol, than either ßVLDL or IDL (approximately 60 µg/ml) which is too little to induce cholesterol 7α-hydroxylase expression. The HDL particle also did not deliver cholesterol to the cells as is reflected by the absence of an effect on intracellular lipid levels and bile acid synthesis, indicating that there may be insufficient supply of regulatory cholesterol to enhance cholesterol 7α-hydroxylase expression. This is in line with previous studies which have shown that apo E-rich HDL is indeed able to stimulate bile acid synthesis in cultured rat hepatocytes (36-38) and that this increase is proportional to the apo E content of the particle (38). In contrast, Whiting et al. reported an increased bile acid synthesis after incubation of cultured rabbit hepatocytes with apo E-free HDL (39).

Our results are in line with *in vivo* studies, showing that feeding a cholesterol-rich diet led to an increased cholesterol 7α-hydroxylase gene expression (25-29). Continuous intravenous infusion of mevalonate, as a source of cholesterol, in control and bile-fistulated rats stimulated cholesterol 7α-hydroxylase enzyme activity, mRNA levels and transcriptional activity (31,32). The latter *in vivo* studies, in which the intestinal route of cholesterol administration is avoided, and our results favour a direct stimulatory mechanism over an indirect mechanism via intestinal malabsorption of different bile acids, as proposed by others (28,33-35). Moreover, Duane (48) showed marked differences in the absorption of different bile acids after cholesterol-feeding in rats, indicating that some potent regulators of bile acid synthesis (chenodeoxycholic acid) escape intestinal entrapment and others do not (cholic acid). This would lead to a scenario where the ratio of cholesterol to bile acids determines the transcriptional activity of the cholesterol 7α-hydroxylase gene, which seems more likely.

Previous reports (13,16-19,49) have shown co-ordinate regulation of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase by the same mediators. This study and those of others *in vivo* (14,15) and *in vitro* (49) demonstrate that both enzymes diverge, however, with respect to regulation by exogenous and endogenous cholesterol, respectively. We showed that strongly increasing the regulatory pool of cholesterol by incubations with lipoproteins and the ACAT-inhibitor CI-1011, did not affect sterol 27-hydroxylase. In contrast, an increasing effect on sterol 27-hydroxylase was found *in vivo* in other species fed a cholesterol-rich diet (15,50) with no concomitant induction (50) or even a decline (15) in cholesterol 7α-

hydroxylase expression. This indicates that species differences exist for both the expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase by cholesterol. Nuclear run-off studies showed increased transcription of cholesterol 7α-hydroxylase by cholesterol-rich lipoproteins, suggesting the presence of a sterol responsive element within it's promoter. These motifs may differ from those in the promoter region of the LDL-receptor and HMG-CoA reductase genes, as cholesterol or metabolite(s) suppress the expression of these genes (51). During the preparation of this work a paper appeared (52) which suggests that it is not cholesterol but its oxidised metabolites which are responsible for stimulating cholesterol 7α-hydroxylase expression. In mice lacking the nuclear oxysterol receptor LXRα, no induction of cholesterol 7α-hydroxylase was found after they were fed a cholesterol-rich diet. In our study, a cholesterol responsive element was localised within the proximal 348 nucleotides of the cholesterol 7α-hydroxylase promoter, which harbours a large amount of different responsive elements to various physiological signals (18,44-46,53). The exact location and nature of this sterol responsive site has yet to be identified. Transcriptional regulation of cholesterol 7α-hydroxylase by exogenous cholesterol has also been reported in H2.35 and HepG2 cells, but regulatory elements were found far more distally (30).

In conclusion, we have shown that circulating cholesterol-laden lipoproteins are not only the substrate for bile acid synthesis, but that cholesterol from these lipoproteins or a metabolite thereof is directly regulatory at the level of cholesterol 7α-hydroxylase gene transcription.

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Chapter 3

Acyl-Coenzyme A:Cholesterol Acyltransferase inhibitor, Avasimibe, stimulates bile acid synthesis and cholesterol 7α-hydroxylase in cultured rat hepatocytes and in *vivo* in rat

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Acyl-Coenzyme A:cholesterol acyltransferase inhibitor, Avasimibe, stimulates bile acid synthesis and cholesterol 7α-hydroxylase in cultured rat hepatocytes and *in vivo* in rat

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Running title: Avasimibe increases bile acid synthesis

ABSTRACT

Acyl-CoA:cholesterol acyltransferase inhibitors are currently in clinical development as potential lipid-lowering and anti-atherosclerotic agents. We investigated the effect of avasimibe (CI-1011), a novel ACAT inhibitor, on bile acid synthesis and cholesterol 7α-hydroxylase in cultured rat hepatocytes and rats fed different diets.

Avasimibe dose-dependently decreased ACAT activity in rat hepatocytes, in the presence and absence of ßVLDL (-93%% and -75% at 10 µM) and reduced intracellular storage of cholesteryl esters. Avasimibe (3 µM) increased bile acid synthesis (2.9 fold), after preincubation with ßVLDL and cholesterol 7α-hydroxylase activity (1.7- and 2.6-fold, with or without ßVLDL), the latter paralleled by a similar induction of its mRNA. Hepatocytes treated with avasimibe showed a shift from storage and secretion of cholesteryl esters to conversion of cholesterol into bile acids.

In rats fed diets containing different amounts of cholesterol and cholate, avasimibe reduced plasma cholesterol (- 52% to -71%) and triglyceride levels (-28% to -62%). Avasimibe did not further increase cholesterol 7α-hydroxylase activity and mRNA in cholesterol-fed rats, but prevented down-regulation by cholate. Avasimibe did not affect sterol 27-hydroxylase and oxysterol 7α-hydroxylase, two enzymes in the alternative pathway in bile acid synthesis. No increase in the ratio of biliary

excreted cholesterol to bile acids was found, indicating that ACAT inhibition does not result in a more lithogenic bile.

Avasimibe increases bile acid synthesis in cultured hepatocytes by enhancing the supply of free cholesterol both as substrate and inducer of cholesterol 7α-hydroxylase. These effects may explain partially the potent cholesterol-lowering effects of avasimibe in rat.

INTRODUCTION

Acyl-coenzyme A:cholesterol acyltransferase (ACAT) is the major enzyme in esterification of cholesterol, a process which plays an important role in different tissues. ACAT, located in the endoplasmic reticulum, is involved in cholesterol absorption in the intestine and in the accumulation of cholesterol in macrophages in the arterial wall. In the liver ACAT is implicated in the storage of cholesteryl esters and the assembly and secretion of very low-density lipoproteins (VLDL) (1). Therefore, ACAT inhibitors may act as hypocholesterolemic and anti-atherosclerotic agents. Recently, a novel class of ACAT inhibitors with improved bioavailability was developed, able to inhibit both intestinal and hepatic ACAT (2). One of these ACAT inhibitors is avasimibe (CI-1011), ([[2,4,6-tris- (1-ethylethyl) phenyl] acetyl]]sulfamic acid, 2,6-bis (1-methyl-ethyl)phenyl ester), which has previously been shown to have cholesterol-lowering activity and to decrease plasma triglyceride levels in animal studies (3).

In addition to the esterification of cholesterol, which leads to its accumulation in tissues and to its secretion in lipoproteins into the circulation, an important cholesterol-metabolizing pathway in the liver is the conversion of cholesterol into bile acids. Bile acid synthesis and secretion in combination with the excretion of free cholesterol into the bile is the major route for the elimination of cholesterol from the mammalian body (4,5). The primary route in bile acid biosynthesis in rats and humans is initiated by 7α-hydroxylation of cholesterol catalyzed by the major rate-limiting enzyme cholesterol 7α-hydroxylase, which is located in the smooth endoplasmic reticulum. This pathway predominantly causes the formation of cholate and chenodeoxycholate (6-8). An alternative pathway in bile acid synthesis is operational as well, contributing considerably to the total bile acid synthesis in humans (9), rats (10), rabbits (11) and in cultured human and rat hepatocytes (12,13). The latter pathway is initiated by the conversion of cholesterol by the

enzyme sterol 27-hydroxylase, which is located in the inner mitochondrial membrane, leading predominantly to the formation of chenodeoxycholate (9,10,12-16). An important enzyme in this pathway is oxysterol 7α-hydroxylase (17,18).

Inhibition of ACAT in the liver is thought to enhance the pool of free cholesterol available for elimination via the bile either directly or after conversion into bile acids. This pool of cholesterol is mostly derived from lipoproteins (19). In addition, a regulatory role within bile acid synthesis has been assigned to cholesterol, which acts either as a substrate or as a factor involved in transcriptional regulation (for review see 20). Differences in cholesterol supply have been shown to modulate cholesterol 7α-hydroxylase activity and gene expression in different ways in various species (21-25) including the rat (26). In this study we investigated the effect of substrate supply after the inhibition of hepatic ACAT by avasimibe on bile acid synthesis in primary cultures of rat hepatocytes and in the rat *in vivo* under various dietary conditions.

Our data indicate that inhibition of ACAT in the liver causes a flow of free cholesterol to supply substrate for bile acid synthesis and provides a pool of regulatory cholesterol inducing cholesterol 7α-hydroxylase, without increasing the lithogenicity of the bile.

MATERIAL AND METHODS

Materials and animals

Avasimibe (CI-1011) was kindly provided by Dr. Krause, Parke-Davis, Ann Arbor, USA. Materials used for the isolation and culturing of rat hepatocytes, and assaying cholesterol 7α-hydroxylase were obtained from sources described previously (27-29). [α-^{32}P]dCTP (3000 Ci/mmol) and [1-^{14}C]-oleate (60mCi/mmol) were obtained from Amersham Life Sciences, Buckinghamshire, UK. 25-[^{3}H]hydroxycholesterol was obtained from NEN Life Science Products (Boston, USA). ßVLDL was obtained from rabbit fed a diet containing 1% (w/w) cholesterol for 1 week. ßVLDL was isolated from plasma by ultracentrifugation at 40000 rpm (285000 x g) in a SW40 rotor for 18 hr at 4°C on a NaCl density gradient: composed of 4 mL plasma (density (ρ) =1.21 with KBr) + 2.6 mL NaCl (ρ=1.063) + 8.6 mL water.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water *ad libitum*. *In vitro* experiments: Two days before the isolation of hepatocytes, the rats were fed a diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands). For the preparation of hepatocytes, the animals were killed between 9 and 10 a.m. *In vivo* experiments: Four days before starting the experiments all rats were fed a control low-cholesterol low-fat (LFC) diet, composed essentially as described by Nishina *et al.* (30) and purchased from Hope Farms, Woerden,

The Netherlands. At the beginning of the experiment (t=0) groups of 8 rats were transferred onto three different diets for 2 weeks. The three semisynthetic diets were: an LFC diet containing basically sucrose and nutrients; a high fat/high cholesterol diet (HFC/0) containing additionally 15% (w/w) cocoa butter and 1% (w/w) cholesterol; and a similar high fat/high cholesterol diet with additionally 0.5% (w/w) cholate (HFC/0.5) to facilitate intestinal uptake of fat and cholesterol and to suppress bile acid synthesis. At the beginning of the second week (t=7 days) the diet of half of the animals in each group was mixed with 0.01% (w/w) avasimibe, which approximately equals a daily dose of 10 mg/kg body weight. Blood samples were taken at t=0, 7 and 14 days. At t=14 days animals were killed and livers were isolated to measure intracellular lipid levels, enzyme activities and mRNA levels. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously (27-29). After a 4-hour attachment period, the cell medium was refreshed with 1.0 mL (6-well plates) or 2.5 mL (dishes) of Williams E (WE) medium supplemented with 10% fetal calf serum (FCS), and cells were incubated for a further 14 hours. Avasimibe, dissolved in DMSO, was added to the culture medium, between 18 and 42 hours of culture age, unless otherwise stated. The final concentration ofDMSO in the medium was 0.1% (v/v). Cells were harvested at the same time after a 42-hour culture period for measuring of cellular lipid, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, and determination of mRNA levels. Cell viability, after culturing with avasimibe, was assessed by ATP measurements and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl bromide (MTT) assays as described previously (29). The latter assay depends on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically.

Quantitation of ACAT activity and measurement of the mass of intracellular and extracellular lipids

Cholesterol esterification was measured by determining the incorporation of [1-^{14}C]-oleate (100 µM, 0.25 µCi) into labeled cholesteryl oleate after the incubation of hepatocytes for 22 h, from 18-40 hours of culture, with different concentrations of avasimibe in the presence or absence of ßVLDL (providing 200 µg cholesterol per mL medium). After 22 h [1-^{14}C]-oleate was added and cells were incubated for another 2 hours at 37°C. Cells were harvested at 42 hours of culture age to measure cholesterol esterification as described previously (31).

To determine the effect of avasimibe on the mass of cellular lipids, the cells were incubated for 24 hours with or without avasimibe or ßVLDL, washed 3 times with cold (4°C) phosphate-buffered saline (PBS) (pH 7.4) and harvested by scraping. The cells thus collected or liver samples from the rats treated with or withoutavasimibe on the different diets were homogenized and samples were taken for measuring the protein content. Lipids were extracted from the cell suspension as described by Bligh & Dyer (32), after the addition of cholesterol acetate (2 µg per sample) as an internal standard. The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 precoated plates as described (29,31). Quantitation of the amounts was performed by scanning the plates with

a Hewlett Packard Scanjet 4c and by integration of the density areas using the computer program Tina version 2.09.

To determine the mass of triglycerides, free cholesterol and cholesteryl ester secreted into the medium, the cells were incubated for 24 hours, from 26 to 50 hours of culture age, in WE medium containing 10% lipoprotein-deficient serum (LPDS) after an 8-hour preincubation period with ßVLDL and extensive washing with WE medium at 37°C to remove extracellular cholesterol. At the end of the incubation period the medium was centrifuged at 12000 rpm (12720 x g) for 30 min at 4°C to remove detached cells and cell debris. The corresponding cells were used for measuring intracellular lipid levels. Extraction and analysis of excreted lipids were the same as those described for the cell suspensions and liver samples.

Lipoprotein analysis

Total plasma cholesterol and triglycerides were measured enzymatically using commercially available kits (Boehringer Mannheim GmbH, Mannheim, Germany). Pooled plasma of two rats was fractionated by FPLC analysis on a 25-mL Superose 6B column (Pharmacia AB, Uppsala, Sweden) and eluted with PBS, pH 7.4 (33).

Quantitation of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by gas-liquid-chromatography (g.l.c.) after a preincubation period of 8 hours (from 18-26 hours of culture age) with ßVLDL (200 µg cholesterol per mL medium), for the following 24 hours culture period from 26-50 hours in WE medium containing 10% LPDS or FCS in the presence or absence of avasimibe as described previously (29).

Determination of biliary bile acid and cholesterol secretion in rats

To study the effects of avasimibe on bile formation, rats were fed an HFC/0.5 diet for two weeks to increase hepatic cholesterol content. After this treatment period, rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere (34). Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. Four days after surgery, i.e., after animals had regained their preoperative body weights rats were transferred onto an HFC/0 diet (to wash-out the cholate) with or without avasimibe (0.01% w/w). After one week of treatment, the connection between both catheters was interrupted and bile was collected for 8 hours in 30-min intervals by means of a fraction collector. Bile volume was determined gravimetrically and samples were immediately stored at -20 °C for later analysis. Bile acids in bile were determined by an enzymatic fluorimetric assay (35). Cholesterol in bile was measured after lipid extraction (32) as described previously (36).

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cell homogenates and isolated liver microsomes were determined essentially according to Chiang (37), measuring mass conversion of cholesterol into 7α- and 27-hydroxycholesterol. In short, 1 to 4 mg of protein of either microsomes or homogenates of cells was incubated in 1 mL of buffer containing 0.1 M potassium phosphate pH 7.2, 50mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v) and 0.015 % (w/w) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS). Twenty µl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-

cyclodextrin was added and the mixture was incubated under agitation for 10 min at 37°C. Then 200 µl of a regenerating system was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min of incubation 60 µl of a stop solution containing 20% (w/v) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. Steroid products were oxidized at 37 °C for 45 min after addition of 100 µL buffer containing 0.1% cholesteroloxidase (w/v) (Calbiochem, USA, #228250), 10 mM potassium phosphate pH 7.4, 1 mM DTT and 20% glycerin (w/w), and the reaction was stopped by the addition of 2 mL ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitril and 30% methanol at a flow rate of 0.8 mL/min. The amount of the product formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

Assay of oxysterol 7α-hydroxylase activity

Enzyme activities of oxysterol 7α-hydroxylase in liver microsomes were determined according to Schwarz (17) measuring mass conversion of 25-[^{3}H]hydroxycholesterol into [^{3}H]cholest-5-ene-3ß,7α,25-triol. In short, 500 µg microsomal protein was incubated with 0.12 µM 25-[^{3}H]hydroxycholesterol in a buffer containing 50 mM Tris-acetate (pH 7.4), 1 mM EDTA, 2 mM DTT, 0.03% (v/v) Triton X-100 and 1.5 mM NADPH for 15 min at 37°C. The reaction was stopped by addition of the addition of 6 mL of methylene chloride. The organic phase was evaporated under a stream of nitrogen. Residues resuspended in acetone were analyzed by thin-layer chromatography in a solvent system containing toluene/ethyl acetate (2:3).

RNA isolation, blotting and hybridization procedures

Isolation of total RNA, and subsequent electrophoresis, Northern-blotting and hybridization techniques were performed as described previously (28,29). The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (28); a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, a 435 bp PstI fragment of hamster HMG-CoA synthase cDNA, and a 2.2 kb EcoRI fragment of rat LDL-receptor cDNA. As controls, a 1.2 kb PstI fragment of hamster ß-actin cDNA, and a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used (29). The actin or GAPDH mRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantitated using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Statistical analysis

Data were analyzed statistically using Student's paired (hepatocytes) or unpaired (rats) t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± S.E.M.

RESULTS

Avasimibe inhibits ACAT activity in cultured rat hepatocytes.

The effect of avasimibe on ACAT activity was measured in hepatocytes in absence or presence of an additional source of cholesterol, in the form of ßVLDL, to increase the intracellular cholesterol pool. Incubation of the rat hepatocytes with avasimibe resulted in a dose-dependent decline in ACAT activity, showing a 75% inhibition at 10 µM. The addition of ßVLDL to the cells resulted in an increase in ACAT activity (2.5-fold) compared to control cells. ACAT inhibition by avasimibe under these conditions decreased the activity dose-dependently to the same level as in control cells (-93%) (Fig 1). The concentrations used in these experiments (up to 10 µM of avasimibe) did not have any adverse effects on cell viability as indicated by measurements of cellular MTT and ATP levels (96 ± 5% and 107 ± 5%, respectively). Data are expressed as a percentage of control and are means ± S.E.M. of independent experiments using hepatocytes from 6 rats.

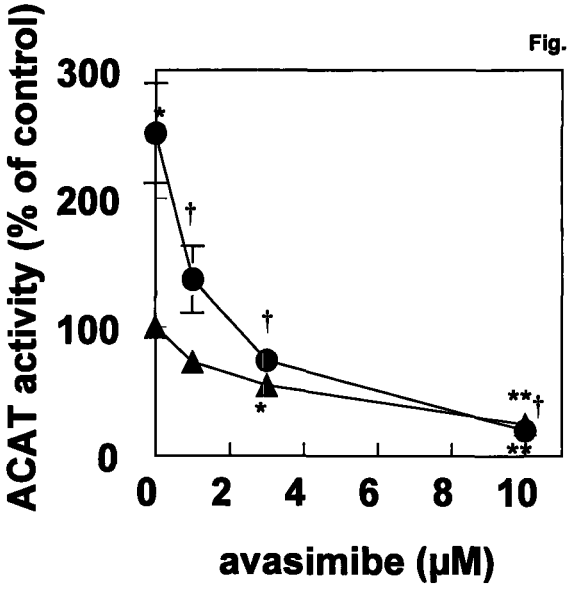

Fig. 1 Effect of avasimibe on ACAT activity
Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, in the presence or absence of avasimibe or ßVLDL (200 µg cholesterol per mL medium). During the last 2 hours of the incubation [1-^{14}C]oleic acid (100 µM, 0.25 µCi) was added to the cells, and at 42 hours of culture age ACAT activity was measured by determining the incorporation of [1-^{14}C]oleic acid into labeled cholesteryl oleate. Values shown are expressed as a percentage of ACAT activity in control incubations and are means ± S.E.M. of independent experiments with hepatocytes of 3 to 5 rats. A significant difference is indicated by symbols (* $P<0.05$ and ** $P<0.005$ compared with control cells (without incubation with avasimibe); † $P<0.001$ compared with the incubation in the presence of ßVLDL without avasimibe).

To investigate the effect of ACAT inhibition by avasimibe on intracellular lipid content the amount of free cholesterol and cholesteryl esters was measured. Incubation with ßVLDL resulted in a 1.8-fold increase in cholesteryl esters in the cells

(Table 1). The addition of 3 µM avasimibe produced a marked decline in cholesteryl ester content in control cells (-41%) and in cells incubated with ßVLDL (-55%).

Table 1. Effect of avasimibe on intracellular lipid levels

	FC	CE
	(% of control)	
Control	100	100
3 µM avasimibe	95 ± 4	59 ± 8*
ßVLDL	98 ± 3	183 ± 15*
ßVLDL +3 µM avasimibe	108 ± 7	83 ± 5*‡ (45)

Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, in the presence or absence of 3 µM avasimibe and an additional source of cholesterol (ßVLDL, 200 µg cholesterol per mL medium). Cells were harvested and cellular cholesteryl ester (CE) and free cholesterol (FC) were measured as described in "Material and Methods". Values are expressed as a percentage of control, and are means ± S.E.M. of independent experiments with hepatocytes from 4-6 rats. In parentheses the effect of avasimibe is given as a percentage of control with ßVLDL. The mean absolute amounts, present in control cells, were 6.4 ± 0.7 µg/mg cellular protein CE and 9.4 ± 1.4 µg/mg cellular protein FC. A significant difference is indicated by symbols (* $P<0.05$, compared with control cells (without avasimibe); ‡ $P<0.001$ compared with the incubation with ßVLDL without avasimibe).

Avasimibe increases bile acid synthesis

To investigate the fate of the free cholesterol which becomes available after ACAT inhibition, bile acid mass production was measured. Cells were preincubated for 8 hours with ßVLDL, to increase the initial intracellular cholesterol content. Incubation with 3 µM avasimibe resulted in a 2.9-fold increase in bile acid mass production (Fig 2). The main bile acids formed were cholic acid and ß-muricholic acid in a ratio of approximately 20:80, which did not change after incubation with the ACAT inhibitor. In the latter experiments bile acid mass production was measured after incubating the cells in WE medium supplemented with 10% LPDS. However, a similar effect of avasimibe on bile acid synthesis was found in medium with 10% FCS (data not shown).

In the cultures incubated with LPDS a cholesterol balance was determined by measuring the total amount of cellular and excreted lipids. Table 2 shows that the increase in bile acid synthesis after incubation with avasimibe is at the expense of the total amount of intracellular and secreted cholesterol mainly contained in the

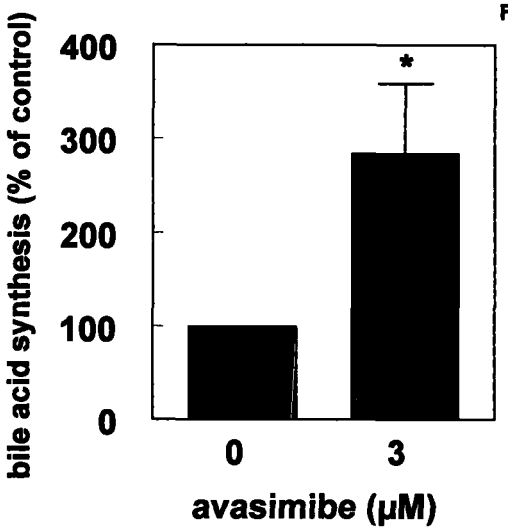

Fig. 2 Effect of avasimibe on mass production of bile acids.
After an 8-hour preincubation period (from 18-26 hours of culture) with ßVLDL (200 µg cholesterol per mL medium), rat hepatocytes were cultured for 24 hours in WE medium containing 10% LPDS in the presence or absence of 3 µM avasimibe. Values shown are expressed as a percentage of bile acid synthesis in control incubations and are means ± S.E.M. of independent experiments with hepatocytes from 4 rats. Absolute synthesis rate in the absence of avasimibe was 4.29 ± 0.66 µg/24hours/mg cell protein. A significant difference between control and treated cells is indicated by an asterisk (P<.05).

Table 2. Effect of avasimibe on cholesterol balance

	Intracellular		Extracellular		total bile
	FC	CE	FC	CE	acids
	(µg/mg cell protein)				
Control	7.8 ± 2.7	6.5 ± 1.4	3.3 ± 0.5	6.1 ± 1.5	3.9 ± 0.6
3µM avasimibe	9.5 ± 3.2* (122)	3.3 ± 0.7* (51)	2.6 ± 0.5* (79)	2.4 ± 0.5* (39)	11.2 ± 2.9* (287)

Rat hepatocytes were incubated in WE medium containing 10% LPDS for 24 hours, from 26-50 hours of culture in the presence or absence of 3 µM avasimibe after a preincubation for 8 hours with ßVLDL (200µg cholesterol per mL medium). Cells and medium were harvested to measure intra- and extracellular free (FC) and esterified (CE) cholesterol and total mass of bile acids. Values are expressed as the amount of cholesterol present as free or esterified cholesterol or as bile acids, and are means ± S.E.M. of independent experiments with hepatocytes from 4 rats. A significance difference between control and treated cells is indicated by an asterisk (P<.05). Values between parenthesis represent data expressed as a percentage of the value in control cells.

cholesteryl ester fraction. Intracellular triglyceride levels were significantly increased (+20 ± 6%) and excretion of triglycerides in these experiments was significantly (P<.05) decreased (-33 ± 6%) (data not shown). Thus, it can be demonstrated, that

when cells are incubated with avasimibe, the flow of cholesterol, which is mostly derived from lipoproteins (ßVLDL) shifts from mainly storage and secretion of cholesterol esters to the conversion of cholesterol into bile acids.

Avasimibe induces cholesterol 7α-hydroxylase activity and mRNA levels

To investigate whether changes in cholesterol 7α-hydroxylase and sterol 27-hydroxylase contribute to the increased bile acid mass production induced by avasimibe, enzyme activities and mRNA levels were determined. Rat hepatocytes were cultured in the absence or presence of ßVLDL and avasimibe. ßVLDL enhanced cholesterol 7α-hydroxylase activity and mRNA, 2.7- and 1.5-fold, respectively (Fig 3). The addition of avasimibe increased cholesterol 7α-hydroxylase enzyme activity and mRNA levels in control cells 2.6- and 2-fold and further increased enzyme activity and the mRNA level in the presence of ßVLDL (1.7- and

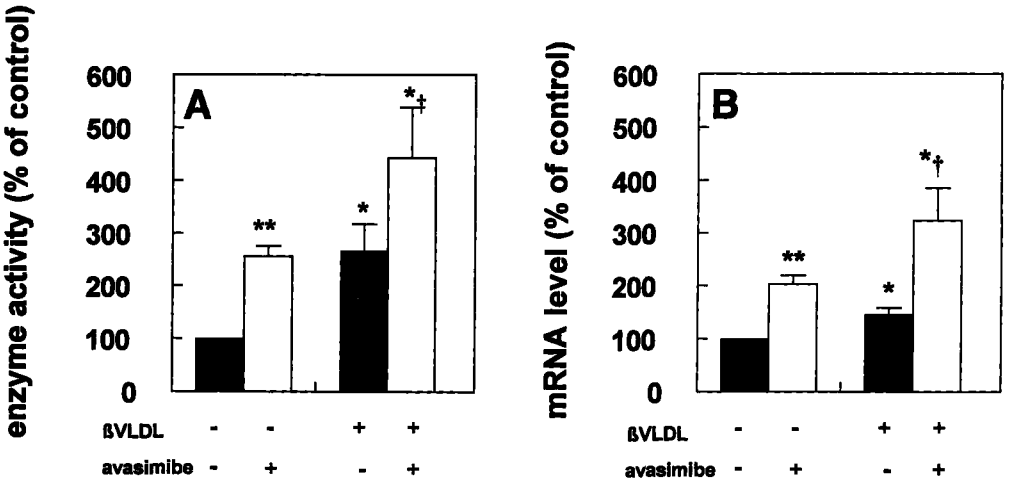

Fig. 3 Effect of avasimibe on cholesterol 7α-hydroxylase activity and mRNA levels

Rat hepatocytes were incubated for 24 hours from 18-42 hours of culture, in the presence or absence of 3 µM avasimibe or ßVLDL (200 µg cholesterol per mL medium). Cells were harvested after 24 hours of incubation to measure cholesterol 7α-hydroxylase activity (Fig 3A) and mRNA levels (Fig 3B). Values shown are expressed as a percentage of enzyme activity or mRNA levels in control cells and are means ± S.E.M. of independent experiments with hepatocytes from 4 to 7 rats. The amount of mRNA was corrected for differences in total RNA applied to the gel, using GAPDH mRNA as an internal standard. Absolute activities of cholesterol 7α-hydroxylase in cell homogenates in the absence of avasimibe were 119 ± 18 pmol/h/mg cell protein. A significant difference between control and treated cells is indicated by symbols (* $P<0.05$; ** $P<0.005$ compared with control cells (without incubation with avasimibe); † $P<0.05$ compared with the incubation with ßVLDL without avasimibe).

2.2-fold, with respect to the incubations containing ßVLDL) (Fig 3). The enhancing effect of avasimibe on cholesterol 7α-hydroxylase mRNA was rapid and significant after as few as 8 hours of incubation, showing a 2-fold increase (data not shown). Neither ßVLDL nor avasimibe had any effect on sterol 27-hydroxylase enzyme activity (data not shown) and mRNA (Table 3).

Since the large decrease observed in ACAT activity may also have consequences for the regulatory cholesterol pool influencing the expression of LDL-receptor and cholesterol synthetic enzymes, mRNA levels of the LDL-receptor and HMG-CoA synthase were measured. These mRNA levels decreased upon incubation with avasimibe, by -28% and -36%, respectively (Table 3). These data indicate that the inhibition of ACAT by avasimibe leads to a moderate down-regulation of genes involved in cholesterol synthesis and LDL-receptor-mediated uptake.

Table 3. Effect of avasimibe on different mRNA levels

	mRNA level (% of control)
cholesterol 7α-hydroxylase	202 ±17**
sterol 27-hydroxylase	103 ± 9
LDL-receptor	72 ± 7*
HMG-CoA synthase	64 ± 7*

Rat hepatocytes were incubated in the presence or absence of 3 µM avasimibe for 24 hours, from 18-42 hours of culture time. mRNA levels were assessed by Northern-blot hybridization and scanning of the resulting phosphor-imager plates, using GAPDH as the internal standard to correct for differences in the amount of RNA applied, as described in "Material and Methods". Data are expressed as a percentage of control and are means ± S.E.M. of independent experiments using hepatocytes from 4 to 7 rats. A significant difference between control and treated cells is indicated by asterisks. (*, $P<0.05$; **, $P<0.01$).

Avasimibe decreases plasma cholesterol and triglycerides in rat

To investigate whether avasimibe also induces cholesterol 7α-hydroxylase *in vivo* we fed rats different semi-synthetic diets with and without 0.01% (w/w) of avasimibe. The diets used were one containing no cholesterol but only basically sucrose and

nutrients (LFC), a high fat/high cholesterol diet (HFC/0) containing additionally 15% (w/w) cocoa butter and 1% (w/w) cholesterol, and a similar high fat/high cholesterol diet with additionally 0.5% (w/w) cholate (HFC/0.5) which further increases plasma cholesterol levels by facilitating the intestinal uptake of fat and cholesterol and suppressing bile acid synthesis. Body weight and food intake did not differ according to groups (data not shown). Total plasma cholesterol levels were increased (1.5-fold) after using the HFC/0 diet as compared with the standard LFC diet and 2.4-fold with the HFC/0.5 diet (Fig 4A). Treatment with 0.01% avasimibe completely prevented increases in total plasma cholesterol and reduced the cholesterol levels to an even significantly lower level than those found in untreated animals on an LFC diet. Triglyceride levels were clearly raised (1.9-fold) in rats on an HFC/0 diet compared to the control LFC diet. The addition of cholate to the diet (HFC/0.5) prevented this increase, and reduced triglyceride levels to levels even below those of control

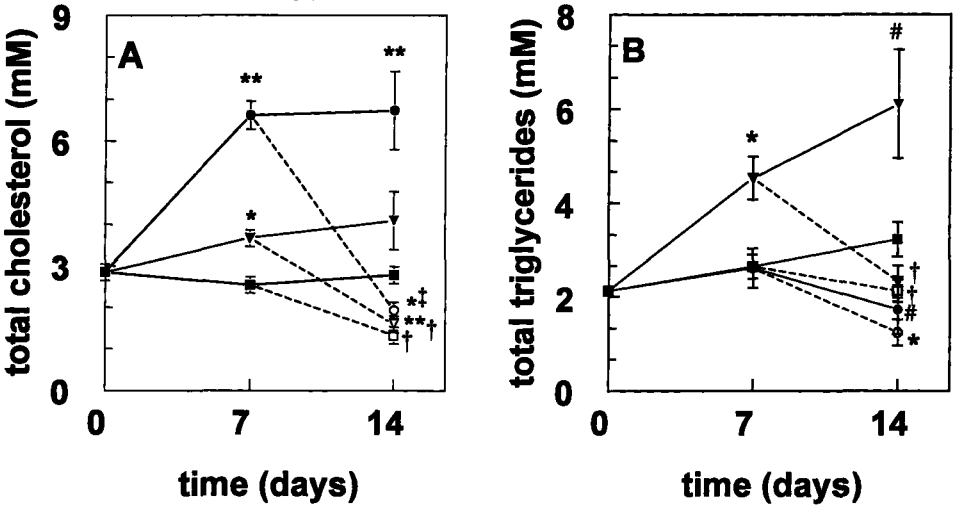

Fig. 4 Effect of avasimibe on plasma cholesterol and triglyceride levels in rat fed different diets. Animals were fed a semi-synthetic diet (LFC, squares) or diets supplemented with 1% cholesterol/15 % cacao butter (HFC/0, triangles) or with 1% cholesterol/15 % cacao butter/0.5% cholate (HFC/0.5, circles) for two weeks (drawn line, closed symbols). Indicated groups received diets containing 0.01% avasimibe during the second week (broken line, open symbols). Blood samples were taken on day 0, 7 and 14. Plasma cholesterol (Fig 4A) and triglycerides (Fig 4B) were measured as described in "Material and Methods". Data represent the mean ± S.E.M. (n=24 on day 0, n=8 on day 7 and n=4 on day 14).A significant difference is indicated by symbols (* P<0.05, ** P<0.01 and $^{\#}$ P=0.055 compared with control LFC diet without avasimibe treatment at the same time point; † P<0.05, ‡ P<0.01 compared with the same diet without avasimibe treatment at the same time point.

untreated animals on the LFC diet. Animals treated with 0.01% avasimibe had reduced triglyceride levels as compared to untreated animals on the same diet (Fig 4B). To determine the effect of avasimibe on the contribution of the various lipoproteins to the changes observed in plasma cholesterol, plasma samples were fractionated by FPLC. The increase in plasma cholesterol induced by dietary cholesterol, and the prevention of these increases by avasimibe was caused predominantly by changes in the VLDL/LDL range. Administration of avasimibe resulted in a decrease in the non-HDL cholesterol in all diets. HDL cholesterol changed depending on the diet fed, showing a decrease in rats fed LFC diet and an increase in the rats fed the HFC/0.5 (data not shown).

Avasimibe decreases hepatic CE levels

To determine the effects of avasimibe on hepatic lipid metabolism levels of liver free and esterified cholesterol and triglycerides were measured. Feeding rats with the cholesterol-rich diet (HFC/0) did not alter the liver free cholesterol levels, but strongly increased the liver cholesteryl ester content (Table 4). The increase in cholesteryl esters was further enhanced in rats on the HFC/0.5 diet, which was now accompanied by a significant rise in liver free cholesterol. Treatment with avasimibe did not affect the cholesteryl ester content with the LFC diet, but the accumulation of cholesteryl esters

was markedly decreased with both the HFC/0 (- 65%) and the HFC/0.5 (- 43%) diets. Liver triglyceride levels were increased in rats on both cholesterol-rich diets (Table 4). Treatment with avasimibe tended to lower hepatic triglyceride levels in rats on the HFC/0 diet, but did not prevent the increase in triglycerides in rats on the HFC/0.5 diet.

Avasimibe stimulates hepatic cholesterol 7α-hydroxylase activity and mRNA levels

Addition of cholesterol to the diet (HFC/0) increased the cholesterol 7α-hydroxylase activity and mRNA levels 2.4- and 2-fold, respectively (Fig 5). This increase was fully abolished by the simultaneous addition of cholate to this diet (HFC/0.5). Treatment with avasimibe did not affect enzyme activity and mRNA levels in rats on the control diet (LFC) or the cholesterol-rich diet (HFC/0). These data indicate that the amount of regulatory cholesterol available after ACAT inhibition in rats on the cholesterol-free diet LFC is too low to establish its effects on cholesterol 7α-hydroxylase. On the

TABLE 4. Effect of avasimibe on hepatic cholesterol and triglyceride content.

Diet	FC	CE	TG
	(μg/mg protein)		
LFC	8.2 ± 0.2	3.2 ± 0.3	9.5 ± 0.3
+avasimibe	8.0 ± 0.4 (96)	3.5 ± 0.7 (110)	10.3 ± 1.3 (109)
HFC/0	9.6 ± 0.8	18.7 ± 2.9**	20.1 ± 3.6*
+avasimibe	8.2 ± 0.4 (86)	6.6 ± 0.6**‡ (35)	12.9 ± 0.9† (64)
HFC/0.5	12.7 ± 0.8**	41.4 ± 0.9**	25.8 ± 3.9**
+avasimibe	11.4 ± 0.4** (90)	23.7 ± 2.7**‡ (57)	24.1 ± 2.1** (93)

Animals were treated with the indicated diet and 0.01% (w/w) avasimibe. In liver homogenates free cholesterol, cholesteryl esters and triglycerides were determined using a h.p.t.l.c. method (see Material and Methods). Data are means ± S.E.M. (n=4). Values between parenthesis represent data expressed as a percentage of value obtained in untreated animals on the same diet. A significant difference is indicated by symbols (*P<0.05 and **P<0.01 compared with control LFC diet without avasimibe treatment; ‡ P<0.01 and † P=0.1 compared with the same diet without avasimibe treatment).

cholesterol-rich diet, HFC/0, the level of cholesterol 7α-hydroxylase expression cannot be further enhanced by increasing the pool of regulatory cholesterol by avasimibe treatment. However, the addition of avasimibe to the HFC/0.5 diet showed a marked increase in cholesterol 7α-hydroxylase activity and mRNA (both 2.9-fold) indicating that ACAT inhibition under conditions of sufficient cholesterol supply can counteract the down-regulation of cholesterol 7α-hydroxylase expression caused by cholate.

Sterol 27-hydroxylase mRNA levels did not change either under the various dietary conditions or after treatment with avasimibe. The oxysterol formed by sterol 27-hydroxylase can be further converted by oxysterol 7α-hydroxylase, which is located in the endoplasmic reticulum (17,18). We also measured the activity of this enzyme to investigate whether expansion of the regulatory pool of cholesterol by avasimibe may have other effects on the alternative pathway. We could not detect significant differences in oxysterol 7α-hydroxylase enzyme activity (Table 5) by feed-

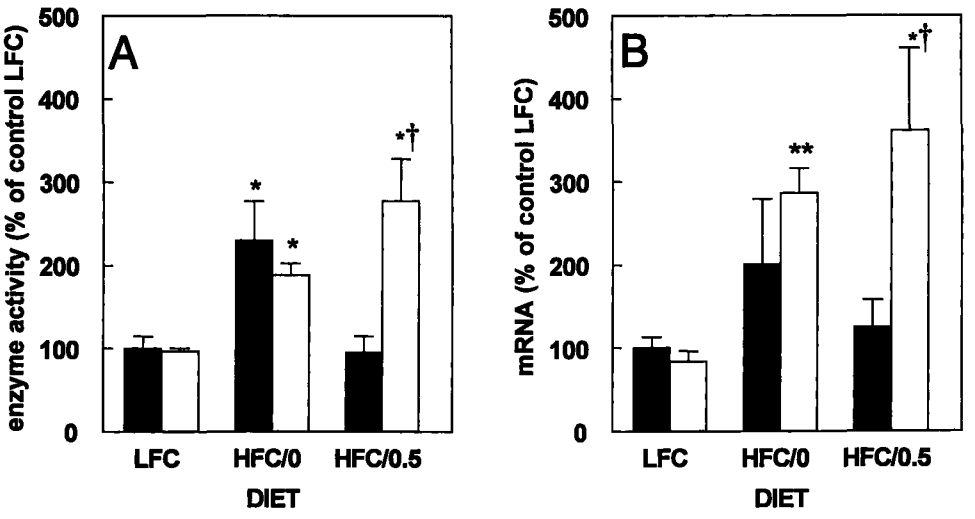

Fig. 5 Effect of avasimibe on cholesterol 7α-hydroxylase activity and mRNA levels in the rat under various dietary conditions.
Cholesterol 7α-hydroxylase activity in liver microsomes (Fig 5A) and mRNA levels (Fig 5B) were measured in rats fed on various diets and treated with or without 0.01% (w/w) avasimibe. Data shown are means ± S.E.M. (n=4 per group). Absolute values for cholesterol 7α-hydroxylase activity were 974 ±143 pmol/h/mg cell protein for control LFC diet. Closed bars: diet only; open bars: diet + avasimibe. A significant difference is indicated by symbols (*,P<0.05 and **,P<0.01 compared with control LFC diet without avasimibe treatment; †,P<0.05 compared with the same diet without avasimibe treatment.)

ing various diets or avasimibe, indicating that two important enzymes in the alternative pathway are not affected by the different diets and inhibition of ACAT. Treatment of rats with dietary cholesterol (HFC/0 and HFC/0.5) decreased LDL-receptor mRNA levels (-40% to -50%), but no further down-regulation was observed after treatment with 0.01% avasimibe (data not shown).

Avasimibe does not affect the lithogenicity index of the bile

To investigate the effect of avasimibe on the overall process of bile acid synthesis, biliary bile acid output was measured in rats fed an HFC/0 diet with or without (0.01 % w/w) avasimibe for 1 week after a pretreatment period for 2 weeks with HFC/0.5 to increase the initial hepatic cholesterol content. After exhaustion of the bile acid pool biliary output of bile acids, which reflects their *de novo* synthesis, was not further increased in the avasimibe treated as compared to control treated group (85.9 ± 17.4 nmol/min/100 g body weight versus 77.8 ± 10.4 nmol/min/100 g body weight,

TABLE 5. Effect of avasimibe on oxysterol 7α-hydroxylase activity

Diet	LFC	HFC	HFC/0.5
		(pmol/mg protein/hr)	
Control	87 ± 11	177 ± 54	158 ± 26
+avasimibe	66 ± 4 (76)	147 ± 25 (83)	213 ± 32 (135)

Oxysterol 7α-hydroxylase activity in liver microsomes was measured in rats fed on various diets and treated with or without 0.01% (w/w) avasimibe. Data shown are means ± S.E.M. (n=4 per group). Values between parenthesis represent data expressed as a percentage of value obtained in untreated animals on the same diet.

respectively) (n=4)). We also determined whether inhibition of ACAT resulted in a quantitatively greater biliary cholesterol secretion as compared to the amount of cholesterol converted to bile acids, which may lead to supersaturation of bile. In the initial 2 hours after interruption of the enterohepatic circulation there was no change in the molar ratio between cholesterol and bile acids excreted after avasimibe treatment as compared to control rats ($4.37*10^{-3}$ ± $0.41*10^{-3}$ versus $4.31*10^{-3}$ ± $0.88*10^{-3}$, respectively (n=4)). Thus, treatment with avasimibe does not lead to supersaturation of the bile.

DISCUSSION

This study demonstrates that avasimibe increases bile acid synthesis by up-regulation of cholesterol 7α-hydroxylase expression in cultured rat hepatocytes both in control cells as well as in cells in which the intracellular cholesterol pool is increased by the addition of ßVLDL. Cholesterol balance experiments showed that the inhibition of ACAT causes a change in the metabolic pathway of cholesterol by reducing lipid secretion and increasing the supply of free cholesterol as a substrate and inducer of cholesterol 7α-hydroxylase resulting in the enhanced production of bile acids. In the rat *in vivo* under conditions of sufficient cholesterol supply (HFC/0.5) avasimibe treatment counteracted the effect of cholate-induced suppression and highly increased the expression of cholesterol 7α-hydroxylase.

Incubation of hepatocytes with avasimibe resulted in a marked decrease in cellular cholesteryl ester content without a concomitant rise in free cholesterol levels. There are several explanations for this. Most importantly, the amount of free cholesterol becoming available after ACAT inhibition in the hepatocytes is rapidly diverted into the bile acid synthetic pathway, as was shown by this study. Furthermore, the high amount of free cholesterol in the cell membranes (38) probably overshadows changes in the free cholesterol content caused by the inhibition of ACAT. However, even small increases in the regulatory cholesterol pool, which is small when compared to the total free intracellular cholesterol mass (31,39), can be monitored by measuring the suppression of the expression of genes involved in cholesterol synthesis and LDL-receptor mediated uptake (39,40). A decrease in the mRNA levels of HMG-CoA synthase and LDL-receptor was observed in cultured hepatocytes after incubation with avasimibe, indicating that the regulatory pool of free cholesterol is indeed enhanced. This is in agreement with previous studies in the human hepatoma cell line HepG2 using the ACAT inhibitor 58-035, showing a marked decrease in LDL-receptor activity (31). *In vivo* in rats fed different diets, the decreased LDL-receptor mRNA levels will contribute to dietary cholesterol-induced increases in plasma cholesterol levels. However, treatment with avasimibe did not further induce LDL-receptor mRNA down-regulation, indicating that there is no further increase in the amount of regulatory cholesterol in the liver.

Other ACAT inhibitors have also been reported to influence bile acid synthesis. The ACAT inhibitor 58-034, DuP-128 and HL-004 showed an increase in bile acid synthesis in rat and hamster hepatocytes and in HepG2 cell, respectively (41-43). In our study, we investigated the biochemical background to the induction of bile acid synthesis by the ACAT inhibitor avasimibe and demonstrated that this is due to an increased expression of cholesterol 7α-hydroxylase both in control cells as well as in cells with a higher cholesterol content due to the addition of ßVLDL. Remarkably, avasimibe even enhanced cholesterol 7α-hydroxylase expression significantly above the level induced by ßVLDL. The increased pool of regulatory cholesterol by incubation with avasimibe or ßVLDL did not affect the expression of sterol 27-hydroxylase in these cells. Even in cells that already had an enhanced cholesterol content through addition of ßVLDL, avasimibe did not evoke any effect on sterol 27-hydroxylase expression. This is in line with the *in vivo* experiments in rats fed different diets, in which we also did not observe any effect on sterol 27-hydroxylase upon diet or avasimibe treatment. Thus, our study and those of others (10,44) indicate that sterol 27-hydroxylase *in vivo* in rat and in rat hepatocytes is

insensitive to cholesterol induction in contrast to the results from experiments with rabbits (11). In addition, oxysterol 7α-hydroxylase was also not affected by ACAT inhibition in the rat fed different diets, indicating that this enzyme is insensitive to cholesterol, as was also observed in mice (18). So, inhibition of ACAT only induces the primary route to bile acid synthesis whereas the alternative route is not affected. In contrast to Schwarz *et al.* (18) we found no decrease in enzyme activity after feeding of a cholic acid containing diet (HFC/0.5) as compared with the HFC/0 diet. The reason for this discrepancy is not known, but may be related to the diet composition or a species difference.

The increased diversion of cholesterol into the bile acid synthetic pathway by the inhibition of hepatic ACAT resulted in a decline in the secretion of cholesteryl esters by the cells, which was accompanied by a decreased excretion of triglycerides. This reflects a suppressed VLDL-lipid excretion as also reported by others (45,46).

In this study, we further evaluated the effects of avasimibe on cholesterol 7α-hydroxylase expression in the rat under various dietary conditions to increase plasma cholesterol and triglyceride levels. To that end, we used semisynthetic casein, sucrose based diets containing different amounts of saturated fat and cholesterol. In these animals avasimibe proved to be a powerful cholesterol-lowering agent, in line with other studies with several rodent models (3) and in cynomolgus monkeys (47). Avasimibe reduced total plasma cholesterol levels in both cholesterol-fed rat models and rats fed a cholesterol-free diet. Even in cholesterol-fed rats in which the plasma cholesterol levels were boosted by dietary cholate, avasimibe lowered the total plasma cholesterol levels below levels obtained by control cholesterol-free diet. An increase in non-HDL cholesterol and a decrease in HDL cholesterol characterize hypercholesterolemia in these studies. Avasimibe reduced non-HDL cholesterol under all dietary conditions. In the rats fed the control and cholesterol-rich diets no increase in HDL cholesterol was found with avasimibe. However, on the cholesterol-rich diet containing cholate avasimibe enhanced HDL cholesterol, like other ACAT inhibitors, which combine a decrease in non-HDL cholesterol with a rise in HDL cholesterol (2,48). The cholesterol-fed rats also showed hypertriglyceridemia, which is prevented by avasimibe in agreement with the hepatocyte experiments, in which concomitantly hepatic secretion of cholesteryl esters and triglycerides was decreased. Increases in plasma triglyceride levels in cholesterol-fed rats (HFC/0 diet), counteracted by avasimibe treatment, reflected the

accumulation of liver triglycerides which is known to be related to the mass production and secretion of VLDL-triglyceride (49-52).

The rat is able to adapt to large fluctuations in sterol input, because of a high basal level of cholesterol 7α-hydroxylase expression sensitive to induction by dietary cholesterol (20,26). Treatment of rats on the cholesterol-free control diet (LFC) with avasimibe did not affect cholesterol 7α-hydroxylase expression, indicating that the amount of regulatory cholesterol available after ACAT inhibition in these rats is too low to evoke effects on cholesterol 7α-hydroxylase. Rats on the cholesterol-rich diet (HFC/0) did have a higher amount of cholesterol in the liver since hepatic cholesteryl esters were strongly increased as compared to the control diet (LFC). This high amount of hepatic cholesterol is an inducer for cholesterol 7α-hydroxylase (20,23,26,53,54) and a substrate for bile acid synthesis (20,21,55). Treatment of rats on the cholesterol-rich diet with avasimibe did not further increase cholesterol 7α-hydroxylase enzyme activity and mRNA levels on the HFC/0 diet, indicating that the level of cholesterol 7α-hydroxylase expression cannot be further enhanced above the level induced on this diet.

Similary, the overall process of bile acid synthesis was not further increased by avasimibe. This is probably due to the high basal level of bile acid synthesis on this diet, which was 3-fold increased as compared with diet containing no cholesterol (56). However, inhibition of hepatic ACAT may still contribute to the maintenance of this high basal level under conditions of a reduced transport of cholesterol to the liver by inhibition of intestinal ACAT. In addition, we found that there was no increase in the biliary excretion of cholesterol, indicating that ACAT inhibition does not result in a more lithogenic bile.

In the case of dietary cholate (HFC/0.5) the amount of cholesterol taken up by the intestine is increased and hepatic bile acid synthesis is suppressed which is reflected by an increase in hepatic cholesteryl esters, and also by an increase in liver free cholesterol levels. Thus, the amount of substrate and regulatory cholesterol required to induce cholesterol 7α-hydroxylase is high, but the situation is compromised by cholate down-regulating bile acid synthesis and cholesterol 7α-hydroxylase expression (13,20,28,53,54), thereby interfering with the enzyme induction of cholesterol 7α-hydroxylase by cholesterol. Treatment with avasimibe reversed this condition by changing the balance between suppression and stimulation in favor of the upregulation of cholesterol 7α-hydroxylase. There are several mechanisms which may contribute to the enhancing effect of avasimibe on

cholesterol 7α-hydroxylase. The increasing amount of intestinal cholesterol, not absorbed due to the inhibition of intestinal ACAT, may interact with bile acids leading to malabsorption and a reduced potential of bile acid-induced feedback as suggested by Björkhem *et al.* (57,58). Further, the increase in the amount of regulatory cholesterol in the liver counteracts the effect of suppression of cholesterol 7α-hydroxylase expression by cholate. Otherwise, avasimibe may by itself reduce cholate uptake in the intestine.

In conclusion, we found that in cultured rat hepatocytes avasimibe increased bile acid synthesis by enhancing the supply of free cholesterol both as a substrate and inducer of cholesterol 7α-hydroxylase. Proper disposal of cholesterol into the bile acid synthetic pathway may contribute to the potent lipid-lowering effects of avasimibe in the rat. However, the primary hypocholesterolemic effect of avasimibe is caused by its decreasing effect on cholesterol absorption by the inhibition of intestinal ACAT, thereby reducing the transport of cholesterol to the liver.

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Chapter 4

Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in rat hepatocytes.

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Cafestol, the cholesterol-raising factor in boiled coffee, suppresses bile acid synthesis by downregulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in rat hepatocytes

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Running title: Cafestol inhibits bile acid synthesis

ABSTRACT

Consumption of boiled coffee raises serum cholesterol levels in man. The diterpenes cafestol and kahweol in boiled coffee have been found to be responsible for the increase. To investigate the biochemical background of this effect, we studied the effects of cafestol and a mixture of cafestol/kahweol/isokahweol (48:47:5 w/w) on bile acid synthesis and cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cultured rat hepatocytes.

Bile acid mass production and cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity were dose-dependently decreased, showing a maximal reduction of -91%, -79%, and -49% respectively, at a concentration of 20 μg/mL cafestol. The decrease in 7α-hydroxylase and 27-hydroxylase activity paralleled well with the suppression of the respective mRNAs, being -79% and -77%, and -49% and -46%, respectively, at 20 μg/mL cafestol. Run-on data showed a reduction in 7α-hydroxylase and 27-hydroxylase gene transcriptional activity after incubation with cafestol. The mixture of cafestol/kahweol/isokahweol was less potent in suppression of bile acid synthesis and cholesterol 7α-hydroxylase. Cafestol (20 μg/mL) had no effect on lithocholic acid 6ß-hydroxylase mRNA, another enzyme involved in bile acid synthesis. LDL-receptor, HMG-CoA reductase, and HMG-CoA synthase mRNAs were significantly decreased by cafestol (-18%, -20%, and -43%, respectively).

We conclude that cafestol suppresses bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and to a lesser extent of sterol 27-hydroxylase in cultured rat hepatocytes, whereas kahweol and isokahweol are less active. We suggest that suppression of bile acid synthesis may provide an explanation for the cholesterol-raising effect of cafestol in humans.

INTRODUCTION

In Scandinavia coffee consumption is associated with elevated levels of serum cholesterol (1,2) and an increased risk of coronary heart disease (3). Scandinavians often drink coffee that is prepared by boiling ground coffee beans with water, instead of the commonly used filtered coffee. The method of brewing is particularly important for the cholesterol-raising effect and the increase in triglyceride levels, i.e. consumption of boiled coffee is associated with a hypercholesterolemic effect, whereas filtered coffee does not increase serum cholesterol levels (4-6). The diterpenes cafestol and kahweol, which are removed upon filtering, were found to be responsible for the cholesterol-raising effect of boiled coffee (7,8). However, the mechanism by which coffee diterpenes cause an increase in serum cholesterol and lipid levels is not well understood. A potential site of action is the liver which plays a pivotal role in the homeostasis of cholesterol.

Removal of low-density-lipoproteins (LDL) from the circulation by the liver is crucial in controling plasma concentrations of LDL cholesterol in humans. In normal subjects more than 70% of this removal takes place via the LDL-receptor (9). The liver plays an important role in synthesis of cholesterol, and the major regulatory and rate-limiting enzyme in this process is 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Furthermore, the liver is the sole organ synthesizing bile acids and conversion of cholesterol into bile acids is the major route for elimination of cholesterol from the mammalian body (10,11). Modulation of bile acid synthesis has been shown to have an effect on serum cholesterol levels. Interruption of the enterohepatic circulation of bile acids by administration of bile acid-binding resins lowers LDL levels and the risk of coronary heart disease in man (12,13). On the other hand, a low bile acid synthetic capacity was found to be an independent risk factor for the incidence of coronary heart disease, and subnormal levels of bile acid synthesis could be correlated to progression of atherosclerosis and coronary mortality in patients heterozygous for Familial Hypercholesterolemia (14). Additionally, an increase in serum levels of LDL and a decrease in bile acid synthetic capacity occur parallel with ageing (15). Animal studies show that genetic factors may influence the responsiveness to dietary cholesterol, as evident from changes in plasma cholesterol concentration, and that this is closely related to the bile acid synthetic capacity (16,17). The rate of bile acid synthesis is therefore considered to be an important regulator of cholesterol homeostasis.

The primary route in bile acid biosynthesis in rats and humans is initiated by 7α-hydroxylation of cholesterol catalyzed by the major rate-limiting enzyme cholesterol 7α-

hydroxylase, which is located in the smooth endoplasmic reticulum. This pathway leads predominantly to the formation of cholate and chenodeoxycholate (18-20). An alternative pathway in bile acid synthesis is operational as well, which contributes considerably to total bile acid synthesis in humans (21) and in cultured human and rat hepatocytes (22,23). This latter pathway is initiated by the enzyme sterol 27-hydroxylase which is located in the inner mitochondrial membrane, leading predominantly to the formation of chenodeoxycholate (21-26).

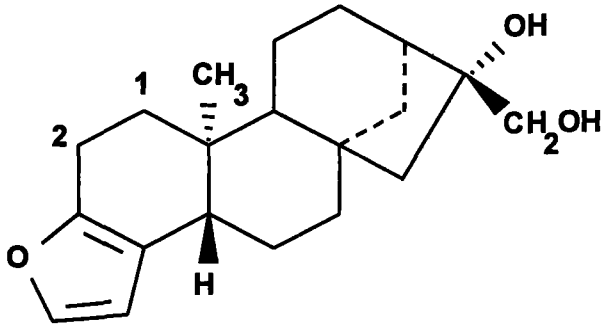

Fig. 1. Structure of cafestol. Kahweol has an additional double bond between C1 and C2.

The coffee diterpenes (Fig. 1) bear structural resemblance to sterols (27). Since sterols, like oxysterols, can have an inhibitory effect on cholesterol 7α-hydroxylase (28,29), bile acid synthesis may be influenced by these compounds. In the light of the effect of the coffee diterpenes on cholesterol levels in humans we studied the effect of these compounds on bile acid synthesis in cultured rat hepatocytes and investigated the mechanism of action.

Our data indicate that cafestol inhibits bile acid synthesis by decreasing cholesterol 7α-hydroxylase and sterol 27-hydroxylase.

MATERIAL AND METHODS

Materials and animals

Materials used for isolation and culturing of rat hepatocytes, and assaying cholesterol 7α-hydroxylase were obtained from sources described previously (30-32). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P]UTP (400 Ci/mmol) and [4-^{14}C]-cholesterol (60 mCi/mol) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire, UK. Cafestol and a mixture of cafestol/kahweol/isokahweol (48:47:5 w/w) were kindly provided by Dr. Huggett, Nestec Research Centre, Lausanne, Switzerland.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water ad libitum. Two days before isolation of hepatocytes, rats were fed a

diet supplemented with 2% cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands) unless otherwise stated. For preparation of hepatocytes, animals were killed between 9 and 10 a.m. Institutional guidelines for animal care were observed in all experiments.
Rat hepatocyte isolation and culture

Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously (30-32). After a 4-hour attachment period, medium of cells was refreshed with 1.0 mL (6-well plates) or 2.5 mL (dishes) of Williams E medium supplemented with 10% fetal calf serum (FCS), and cells were incubated for a further 14 hours. Coffee diterpenes, dissolved in DMSO, were added to the culture medium of cells after this period, between 18-42 hours of culture age, unless otherwise stated. The final concentration of DMSO in the medium was 0.1% (v/v). Cells were harvested at the same time after a 42-hour culture period for measurement of cellular lipid, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, and determination of mRNA levels and transcriptional activity. Cell viability, after culturing with the coffee diterpenes, was assessed by ATP measurements (33) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl bromide (MTT) assays. This assay depends on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells, to a blue formazan product which can be measured spectrophotometrically. The assay was performed essentially as described by De Vries et al. (34). In short, parallel with the various incubations, cells were cultured on 12-wells plates ($5x10^5$ cells/well) in 0.5 mL medium containing coffee diterpenes. At the end of the incubation period, 55 μL of MTT solution (5 mg MTT/mL PBS) was added to each well for 2 hours. The medium was aspirated, and 1 mL 100% DMSO was added to solubilize the formazan crystals. Absorbance at 545 nm was measured immediately.

Quantitation of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by gas-liquid-chromatography (g.l.c.) after a preincubation period of 8 h (from 18-26 hours of culture age), during the following 24 h culture period from 26-50 hours in the absence or presence of coffee diterpenes as described previously (30).

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity in homogenates of cultured rat hepatocytes were measured as described previously (22, 23, 32). Microsomes were isolated as described previously (31). [^{14}C]-labeled products were analyzed by thin layer chromatography, and the amount of [^{14}C]-7α-hydroxycholesterol and [^{14}C]-27-hydroxycholesterol were quantitated by scraping off and counting of the spots containing this product, using the [^{4}C]-cholesterol input as a recovery standard. Blank values, determined by running parallel incubations without a NADPH-generating system, were subtracted before calculating enzyme activity.

RNA isolation, blotting and hybridization procedures

Isolation of total RNA, and subsequent electrophoresis, Northern-blotting and hybridization techniques were performed as described previously (23,30). The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region (30); a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, kindly provided by Dr. J. Strauss (35). A 0.7 kb EcoRI fragment of pFR29-3 containing the cDNA for hamster lithocholic acid 6ß-hydroxylase,

kindly provided by Dr. G.Gil (36), a 773bp HindIII fragment of hamster HMG-CoA reductase cDNA (37), a 435bp PstI fragment of hamster HMG-CoA synthase cDNA (38), and a rat LDL-receptor cDNA (39). As controls a 1.2 kb PstI fragment of hamster β-actin cDNA, and a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were used. The actin or GAPDH mRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels werequantitated by Phosphor-imager (Fuji Fujix BAS 1000) analysis.

Nuclear run-on studies

Nuclear run-on studies were conducted essentially as described in ref. 40.

Hybridization - Target DNA, being 5 μg of plasmid material containing cDNA sequences of rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, hamster actin, rat GAPDH (see the above) and the empty vector pUC19, were slot-blotted onto strips of Hybond-N^+ filter (Amersham), and cross linked with 0.4 N NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described above, and hybridized with the labeled RNA for 36 hours in the same buffer. Labeled RNA was generated by incorporation of [^{32}P]-UTP into nascent RNA, using isolated nuclei from cells which had been cultured with or without cafestol for 24 hours of culture time. After hybridization, the various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to a Fuji imaging plate type BAS-MP for 3-5 days. Quantitation of relative amounts of transcribed mRNA was performed using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version

Measurement of the mass of intracellular triglycerides, cholesterol and cholesteryl esters

After a 24-hour incubation period, with or without coffee diterpenes cells were washed 3 times with cold phosphate-buffered saline (pH 7.4). Thereafter cells were harvested by scraping, and homogenized. Samples were taken for measurement of protein content. Lipids were extracted from the cell suspension as described by Bligh & Dyer (41), after addition of cholesterol acetate (2 μg per sample) as an internal standard. The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 precoated plates as described (42). Quantitation of the amounts was done by scanning the plates with a Shimadzu (Kyoto, Japan) CS910 chromatograph scanner at 380 nm, and areas under the curves were integrated by using a data processor (Shimadzu).

Statistical analysis

Data were analyzed statistically using Student's paired t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± SEM.

RESULTS

Effect of coffee diterpenes on bile acid synthesis

Bile acid mass production was measured over a 24-hour incubation period after a preincubation of 8 hours as described in "Methods". Incubation of the rat hepatocytes

with cafestol or a mixture of cafestol/kahweol/isokahweol (48:47:5 w/w) alone resulted in a dose-dependent decline in bile acid mass production, showing a 91 ± 5% and 68 ± 3% inhibition, respectively at 20 μg/mL (Fig. 2). The main bile acids formed were cholic acid and ß-muricholic acid in a ratio of approximately 20:80 which did not change after incubation with coffee diterpenes. The concentrations used in these experiments at 20 μg/mL of cafestol or the mixture did not have adverse effects on cell viability as shown by measurements of MTT (95 ± 4% and 102 ± 7%, respectively) and of ATP (93± 11% and 110 ± 12%, respectively). Data are expressed as percentage of control and are means ± SEM of independent experiments using hepatocytes from 3 to 4 rats.

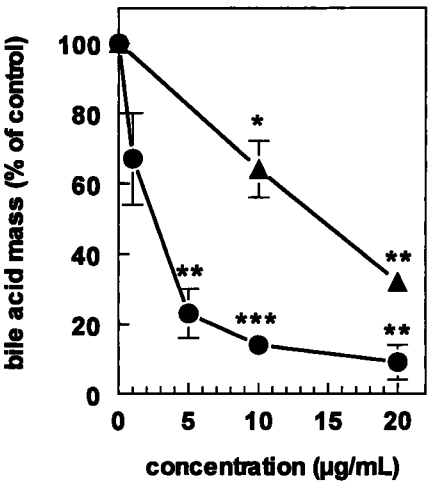

Fig. 2 Effect of cafestol or a mixture cafestol/kahweol/ isokahweol on mass production of bile acids
After a 8-hour preincubation period (from 18-26 hours of culture), rat hepatocytes were cultured for 24 hours (between 26-50 hours of culture) in presence or absence of different concentrations (0-20 μg/mL) of the diterpenes cafestol (circles) or a mixture (triangles). Values shown are expressed as percentage of bile acid synthesis in control incubations and are means (± SEM) of independent experiments with hepatocytes from 3 to 4 rats. Absolute synthesis rate in the absence of coffee diterpenes was 2.06 ± 0.31 μg/24h/mg cell protein. A significant difference between control and treated cells is indicated by an asterix (*, $p<0.05$; **, $p<0.005$; ***, $p<0.001$).

Effect of coffee diterpenes on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels.

To assess the level at which coffee compounds decrease bile acid mass production, enzyme activity and mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were determined. Rat hepatocytes were cultured in the presence or absence of cafestol or the mixture. Fig. 3a shows that there is a dose-dependent decrease in cholesterol 7α-hydroxylase activity with a maximal suppression of -79 ± 3% at a concentration of 20 μg/mL of cafestol. The decrease in cholesterol 7α-hydroxylase activity paralleled well with the decrease in mRNA, being -77 ± 4% at a concentration of 20 μg/mL of cafestol. The 7α-hydroxylase mRNA levels of cells incubated with different concentrations of the mixture also showed a significant decline, but to a lesser extent, with a maximal suppression of -31 ± 8% at 20 μg/mL of the mixture (Fig. 3b).

This suggests that cafestol is the most potent compound and that kahweol and isokahweol are less active. The suppressing effect of cafestol on cholesterol 7α-hydroxylase mRNA was rapid and detectable already after 4 hours of incubation (-43 ± 9%) with 10 μg/mL of cafestol (data not shown). In addition to the effect of cafestol on cholesterol 7α-hydroxylase mRNA and enzyme activity after cell incubation, the compound also had a direct inhibitory effect when added in cholesterol 7α-hydroxylase

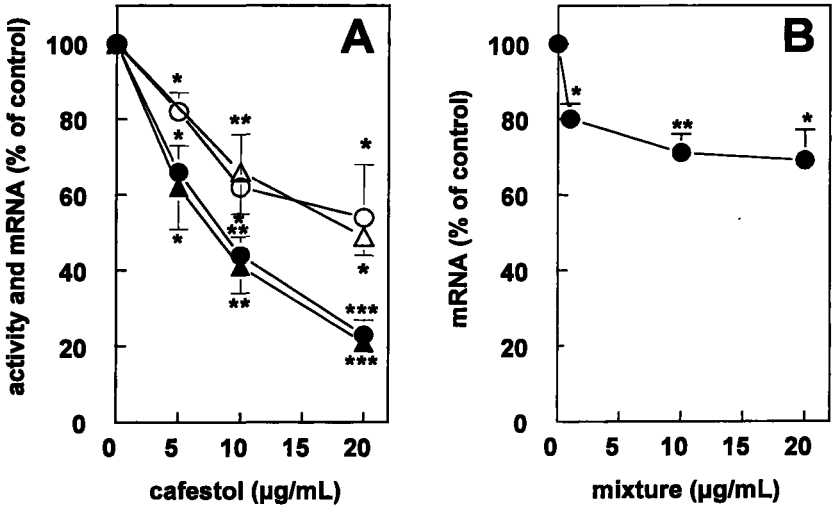

Fig. 3. Effect of cafestol or a mixture of cafestol/kahweol/isokahweol on cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity and mRNA levels
Rat hepatocytes were incubated for 24 hours, from 18-42 hours of culture, in presence or absence of different concentrations of cafestol (3a) or a mixture (3b) (0-20 μg/mL). Cells were harvested after 24 hours of incubation to measure cholesterol 7α-hydroxylase (closed symbols) and sterol 27-hydroxylase (open symbols) activity (triangles) and mRNA levels (circles). Values shown are expressed as percentage of enzyme activity or mRNA levels in control cells and are means (± SEM) of independent experiments with hepatocytes from 4 to 8 rats. The amount of mRNA was corrected for differences in total RNA applied to the filter, using GAPDH mRNA as an internal standard. Absolute activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cell homogenates in the absence of diterpenes were 291 ± 57 and 68 ± 4 pmol/h/mg cell protein, respectively. A significant difference between control and treated cells is indicated by an asterix (*, $p<0.05$; **, $p<0.005$; ***, $p<0.001$).

enzyme activity assays. In homogenates of freshly isolated rat hepatocytes cholesterol 7α-hydroxylase activity was dose-dependently decreased with a maximal suppression of -86 ± 2% at a concentration of 20 μg/mL cafestol. This was in agreement with experiments using isolated rat liver microsomes (Table 1).

Next to the effect on cholesterol 7α-hydroxylase, cafestol also caused a significant ($p<0.05$) and dose-dependent decrease in sterol 27-hydroxylase activity and mRNA levels, being -49 ± 5% and -46 ± 14%, respectively at concentrations of cafestol of 20 μg/mL (Fig. 3a). Cafestol did not have a direct inhibitory effect on sterol 27-hydroxylase activity in homogenates of freshly isolated rat hepatocytes (data not shown). In contrast, mRNA levels of the lithocholic acid 6ß-hydroxylase and of the mRNAs of the house-keeping genes actin and GAPDH did not change significantly upon incubation with 20 μg/mL of cafestol (data not shown).

Table 1: Effect of cafestol on cholesterol 7α-hydroxylase activity in cellhomogenates and microsomes

cafestol (μg/mL)	homogenates (% of control)	microsomes (% of control)
0	100	100
10	30 ± 13**	19 ± 8**
20	14 ± 2***	12 ± 0

*Directly after hepatocyte isolation, a portion of the hepatocytes was washed and frozen in liquid N_2. Homogenates were prepared from these freshly isolated cells and cholesterol 7α-hydroxylase activity was determined in these homogenates. Alternatively, as described in "Methods" isolated rat liver microsomes from rats fed on chow diet were used. Data shown are expressed as percentage of control and are means ± SEM or range from 2 to 4 experiments. The absolute values for cholesterol 7α-hydroxylase activity was 1380 ± 358 pmol/h/mg cell protein in homogenates and 1805 ± 543 pmol/h/mg cell protein in rat liver microsomes. Significant differences are indicated with an asterix (**, $p<0.005$; ***, $p<0.001$).*

Effect of cafestol on the transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

To further examine the mechanism of suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA level, nuclear run-on studies were conducted using nuclei isolated from rat hepatocytes which were incubated in presence or absence of cafestol for 24 hours. [α^{32}P]-labeled total RNA was hybridized to cDNAs for rat cholesterol 7α-hydroxylase, rat sterol 27-hydroxylase, rat GAPDH, and hamster actin. The latter two served as transcriptional activity controls between the different samples and specific transcriptional activity is expressed relative to that of actin. After incubation with 10 μg/mL cafestol there is a significant decrease in cholesterol 7α-hydroxylase and sterol 27-hydroxylase transcriptional activity of -55 ± 13% and -39 ± 6%, respectively (Fig. 4),

well in line with the suppression of the respective mRNA levels at this concentration (-56 ± 5 and -33 ± 12%, respectively).

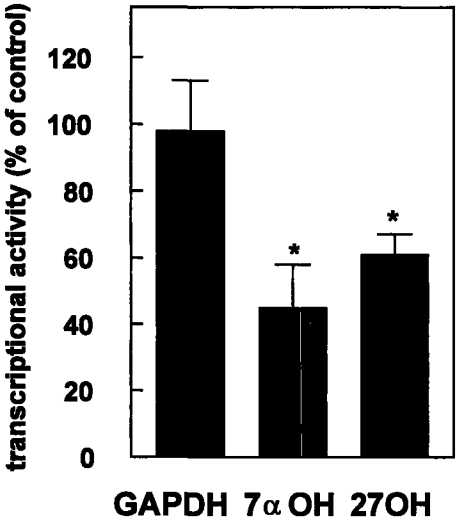

Fig. 4. Effect of cafestol on transcriptional activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

Transcriptional activity of different genes in nuclei isolated from hepatocytes incubated with 10 μg/mL cafestol for 24 hours between 18-42 hours of culture time. Hepatocytes were harvested simultaneously with untreated cells after this period for preparation of nuclei. Transcriptional activity of the different genes was determined by nuclear run-on assays as described in "Methods". Data are expressed as transcriptional activity relative to that of actin, and as percentage of control cells (no cafestol added). Each value represents a mean ± SEM of 3 to 4 independent experiments. A significant difference ($p<0.05$) between control and treated cells is indicated by an asterix.

Effect of cafestol on intracellular lipids and mRNA levels of the LDL-receptor, HMG-CoA reductase, and HMG-CoA synthase

Since such a large decrease in bile acid synthesis may have consequences for the level of intracellular cholesterol, we determined the amount of free and esterified cholesterol in hepatocytes cultured for 24 hours with different amounts of cafestol. The amount of free and esterified cholesterol and of triglycerides, however, did not change significantly upon incubation with 20 μg/mL cafestol (data not shown). Probably these changes are too small to be detectable. Another sensitive measure to detect changes in the regulatory free cholesterol pool is measurement of LDL-receptor mRNA and mRNAs of enzymes involved in cholesterol synthesis, like HMG-CoA reductase and HMG-CoA synthase (43). Table 2 shows that LDL-receptor and HMG-CoA reductase mRNA levels were mildly but significantly decreased (-18 ± 8% and -20 ± 5%, respectively) upon incubation with 20 μg/mL of cafestol, whereas the mRNA of HMG-CoA synthase was clearly suppressed (-43 ± 10%). These data indicate that inhibition of bile acid synthesis by cafestol leads to down-regulation of genes involved in cholesterol synthesis and LDL-receptor-mediated uptake.

Table 2: Effect of cafestol on different mRNA levels

mRNA level	(% of control)
LDL-receptor	82 ± 8*
HMG-CoA reductase	80 ± 5*
HMG-CoA synthase	57 ± 10*

Rat hepatocytes were incubated in presence of absence of cafestol (20 µg/mL) for 24 hours, from 18 to 42 hours of culture time. Cells were harvested after 42 hours, and mRNA levels of the LDL-receptor, HMG-CoA reductase, and HMG-CoA synthase were assessed by Northern-blot hybridization and scanning of the resulting phosphor-imager plates, using GAPDH as internal standard to correct for differences in the amount of RNA applied, as described in "Material and Methods". Data are expressed as percentage of control and are means ± SEM of independent experiments using hepatocytes from 5 to 7 rats. A significant difference ($p<0.05$) between control and treated cells is indicated by an asterix.

DISCUSSION

In this study we have investigated the effect of cafestol and a mixture of cafestol/kahweol/isokahweol (48:47:5 w/w) on bile acid synthesis. Cafestol suppressed bile acid synthesis by a direct inhibitory effect on cholesterol 7α-hydroxylase activity and by down-regulation of mRNA of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. The decrease in mRNA levels is due to a decline of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription, as shown by nuclear run-on assays. Elevation of the initial level of bile acid synthesis by feeding rats chow supplemented with 2% cholestyramine prior to isolation of the hepatocytes (22, 23, 30) was not found to be obligatory to observe down-regulation of bile acid synthesis by coffee diterpenes. Similar results as reported in this paper were obtained using rats fed on control chow (data not shown). Simultaneous with the decline in bile acid synthesis LDL-receptor, HMG-CoA reductase, and HMG-CoA synthase mRNA levels were down-regulated.

Cafestol suppressed bile acid synthesis more potently (2 to 4 fold) than the mixture at the same concentration. In fact the mixture appeared to counteract the effects of cafestol on bile acid synthesis and cholesterol 7α-hydroxylase mRNA level. We conclude therefore that cafestol is the active compound and suggest that kahweol and isokahweol are less or not active. In an intervention study in man Weusten-Van der Wouw et al. (8), found that both oil from Arabica beans, which contains both cafestol

and kahweol, and Robusta oil, which contains cafestol but negligible amounts of kahweol, increased serum cholesterol in healthy volunteers to a similar extent. The latter findings also suggest the involvement of cafestol in raising cholesterol levels, but do not really exclude an additional role for kahweol.

Two different modes of inhibition of bile acid synthesis were found in our study. On the one hand cafestol suppressed bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene transcription which caused a decrease in mRNA levels and activity. Since cafestol resembles sterols, it is conceivable that inhibition of gene transcription by cafestol is regulated via as yet unidentified sequences within the cholesterol 7α-hydroxylase and sterol 27-hydroxylase promoter. On the other hand bile acid synthesis can be affected by a direct inhibitory effect of cafestol on cholesterol 7α-hydroxylase activity. In line with this we found that bile acid mass production is inhibited to a stronger extent than can be explained by suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase alone. In former studies the magnitude of suppression of these parameters was comparable with mediators which do not have a direct inhibitory effect on enzyme activity in the assay, e.g. bile acids and insulin (23,30,40). Based on the structural similarity, coffee diterpenes may act as direct inhibitors like oxysterols, which have been also reported to inhibit cholesterol 7α-hydroxylase activity (28,29). Whether these two different ways of inhibition are linked remains obscure. No evidence was obtained, however, that cafestol has a general effect on cytochrome P-450 enzymes involved in bile acid synthesis, since lithocholic acid 6ß-hydroxylase mRNA was not significantly affected upon addition of cafestol.

Measurement of cellular lipid levels did not show significant rises in cellular free or esterified cholesterol after incubation with cafestol. High amounts of free cholesterol present in the membranes of cells (44) probably overshadow changes in free cholesterol caused by down-regulation of bile acid synthesis by cafestol. Furthermore, an excess of free cholesterol in hepatocytes is rapidly converted into cholesteryl esters (42). Since the decrease in bile acid synthesis was not accompanied by an increase in cholesteryl esters, it is possible that inhibition of bile acid synthesis by cafestol leads to an enhanced production and secretion of very-low-density-lipoprotein (VLDL) particles or biliary cholesterol excretion to remove cholesterol from the cell. However, we found no increase in apolipoprotein B secretion after incubation with cafestol (data not shown). The latter data are in line with observations in CaCo-2 cells, which also did not show a difference in mass of intracellular cholesterol after incubation with cafestol and which

showed decreased rates of secretion of cholesteryl esters and triacylglycerol in these cells, representative for secretion of chylomicrons, in the presence of cafestol (27). Another way to maintain intracellular cholesterol homeostasis during inhibition of bile acid synthesis by cafestol is by down-regulation of cholesterol synthesis and LDL-receptor-mediated uptake. Indeed, a mild but significant suppression of the mRNA levels of the LDL-receptor and HMG-CoA reductase, and a marked decrease in the HMG-CoA synthase mRNA level was found. It is well known that the regulatory free cholesterol pool plays an important role in this regulation and that this pool is small as compared with the total free intracellular cholesterol mass (42-45). Subtle increases in intracellular cholesterol have been shown to prevent processing of sterol regulatory element binding proteins (SREBP), and are shown to be involved in this down-regulation of gene transcription of the LDL-receptor, HMG-CoA reductase, and HMG-CoA synthase genes (46, 47). A modest decrease of LDL-receptor and HMG-CoA reductase mRNA levels by dietary cholesterol has also been shown *in vivo* in the rat (48). In addition, Molowa and Cimis showed that in the human hepatoma cell-line HepG2 both HMG-CoA reductase and LDL-receptor mRNA levels were only moderately down-regulated by LDL as compared to extrahepatic cells (49). In different studies both *in vitro* (50) and *in vivo* (48) it is demonstrated that HMG-CoA reductase can be regulated at different levels, showing only a small decrease in mRNA level, despite the fact that hepatic cholesterol synthesis is largely suppressed. HMG-CoA reductase and HMG-CoA synthase mRNA are coordinately regulated, showing a larger effect on the latter mRNA (38), which is also found in our study. Similarly, in the case of high suppression of hepatic cholesterol synthesis only modest down-regulation of LDL-receptor mRNA can be observed in rat (48). Since the role of the LDL-receptor in controling the cholesterol balance in rats is small in contrast to humans (45). On the other hand, based on the structural similarity of cafestol with oxysterols, a direct effect of cafestol on transcription of the HMG-CoA reductase, HMG-CoA synthase, and LDL-receptor genes can not be excluded. The down-regulation of LDL-receptor mRNA by cafestol in this study is comparable in magnitude to the decrease in LDL uptake in the human hepatoma cell-line HepG2 and human skin fibroblasts reported by others (27).

In conclusion, a decreased bile acid synthesis and down-regulation of the LDL-receptor may form an explanation for the rise in serum cholesterol in humans after consumption of boiled coffee.

ACKNOWLEDGEMENTS

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Chapter 5

Cafestol, the cholesterol-raising factor in boiled coffee, increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis.

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Conditionally accepted

Cafestol, the cholesterol-raising factor in boiled coffee, increases serum cholesterol levels in apolipoprotein E*3-Leiden transgenic mice by suppression of bile acid synthesis

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Running title: Cafestol inhibits bile acid synthesis

ABSTRACT

Cafestol, a diterpene present in unfiltered coffee brews, potently increases serum cholesterol levels in humans. So far, no suitable animal model has been found to study the biochemical background of this effect. We determined the effect of cafestol on serum cholesterol and triglycerides in different strains of mice and studied subsequently the mechanism of action in apoE*3-Leiden transgenic mice.

Apolipoprotein (Apo) E*3-Leiden, heterozygous LDL receptor knock-out (LDLR+/-) or wild type C57Bl/6 (WT) mice were fed a high (0.05% w/w) or low (0.01% w/w) cafestol diet or a placebo diet for 8 weeks. Standardized to energy intake, these amounts equal 40, 8 or 0 cups of unfiltered coffee per 10 MJ per day in humans. In apoE*3-Leiden mice, serum cholesterol was increased by 33% (3.46 mmol/L; 95%CI [1.62;5.30]) on the low and by 61% (6.35 mmol/L; 95%CI [4.47;8.22]) on the high cafestol diet. In LDLR+/- and WT mice, the increases were 20% (0.85 mmol/L; 95%CI [-0.25;1.94]) and 24% (0.62 mmol/L; 95%CI [0.34;0.90]), respectively, on the low cafestol diet, and 55% (2.37 mmol/L; 95%CI [0.73;4.01]) and 46% (1.21 mmol/L; 95%CI [0.92;1.21]), respectively, on the high cafestol diet. The

increase in total cholesterol was mainly due to a rise in VLDL and IDL cholesterol in all three mouse strains.

To investigate the mechanism of the cholesterol-raising effect, apoE*3-Leiden mice were fed a high cafestol or a placebo diet for 3 weeks. Cafestol suppressed enzyme activity and mRNA levels of cholesterol 7α-hydroxylase by 57% and 58% (both $p<0.05$), respectively. mRNA levels of enzymes involved in the alternative pathway of bile acid synthesis i.e. sterol 27-hydroxylase and oxysterol 7α-hydroxylase were reduced, by 32% ($p<0.05$) and 48% ($p<0.005$), respectively. The total amount of bile acids secreted in feces was decreased by 41%. Cafestol did not affect hepatic free and esterified cholesterol, but it decreased LDLR mRNA levels by 37% ($p<0.05$). VLDL particles contained a three times higher amount of cholesteryl esters, indicative for the secretion of a ß-VLDL-like particle. This was confirmed by a decreased VLDL triglyceride production, as measured by the increase in triglycerides after Triton injection, in mice treated with cafestol (35.1±13.8 μmol/h/kg) compared to placebo treatment (63.1±17.5 μmol/h/kg). This was a result of a reduction in hepatic triglyceride content by 52% ($p<0.05$).

In conclusion, cafestol increases serum cholesterol levels in apoE*3-Leiden transgenic mice by suppression of the major regulatory enzymes in the bile acid synthesis pathways, leading to decreased LDLR mRNA levels and increased secretion of cholesterol esters by the liver. In analogy, we suggest that suppression of bile acid synthesis may provide an explanation for the cholesterol-raising effect of cafestol in humans.

INTRODUCTION

Unfiltered coffee brews markedly increase serum cholesterol levels in humans. The responsible compounds for this effect are cafestol and kahweol, two diterpenes that are present in coffee beans (1). From our experiments, cafestol appeared to be by far the most potent compound (2). We estimated that each 10 mg of cafestol ingested per day raises serum cholesterol levels by 0.13 mmol/L (3). In humans, about 80% of the rise in total cholesterol is accounted for by low density lipoprotein (LDL) cholesterol, and the rest is due to a rise in very low density lipoprotein (VLDL) cholesterol (4).

The mechanism by which coffee diterpenes influence lipid metabolism is largely unknown. Recently, we reported that cafestol suppressed bile acid synthesis in cultured rat hepatocytes by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase (5). Suppression of bile acid synthesis will lead to an increased pool of regulatory cholesterol, resulting in a decreased expression of the hepatic LDL-receptor. This may provide an explanation for the cholesterol-raising effect of cafestol in humans (5). The availability of an animal model to study this hypothesis *in vivo* would be of great value, since it may help us to validate our *in vitro* experiments and eventually to discover the metabolic control points of cafestol. However, in previous studies, various animal models like hamsters (6-8), rats (6,9), gerbils (8), Cebus, Rhesus and African green monkeys monkeys (10) did not respond to cafestol and kahweol as humans do, regardless of the dosage, the mode of administration or the duration of the treatment. Differences in the absorption and/or metabolism of coffee diterpenes, or in their effects on lipoprotein metabolism, might underlie the negative results in a range of animal species.

We here studied the effects of cafestol and kahweol on serum lipoproteins in apolipoprotein E*3-Leiden (apoE*3-Leiden) transgenic mice, in heterozygous LDL receptor deficient (LDLR+/-) mice, and in wild type (WT) C57Bl/6 mice. We chose to use transgenic mice over-expressing human apoE*3-Leiden, since these mice are highly susceptible to diet-induced hyperlipoproteinemia primarily due to a partial defect in hepatic uptake of remnant lipoproteins (11,12). Since it was anticipated from our studies in cultured rat hepatocytes that cafestol would have an indirect effect on the expression of the LDL-receptor (5), experiments were also performed with LDLR+/- mice. Lipoprotein profiles from LDLR+/- mice are more similar to humans than those from WT mice, which transport most of their cholesterol in the HDL lipoprotein fraction (13). The cholesterol-raising effect of cafestol appeared to be most pronounced in apoE*3-Leiden transgenic mice, allowing us to investigate the mechanism of the cholesterol-raising effect of cafestol in this mouse strain.

MATERIAL AND METHODS

Animals, housing and diet

Twenty four female apoE*3-Leiden mice (mean age 19 ± 5 weeks), 24 female LDLR+/- mice (mean age 32 ± 2 weeks) and 24 female WT C57Bl/6 mice (mean age 19 ± 5 weeks) were held under standard conditions in Macrolon type III cages housing 4 animals. All cages were

placed under filtertops to prevent infection. They were maintained on 12-h dark and 12-hlight cycles and were allowed free access to food and water. Body weight of the mice and the consumption of diet and water were recorded weekly during the whole experimental period. Mice were fed a common challenge diet enriched with saturated fat and cholesterol (18.2 MJ/kg) containing per 100 g: cacao butter 15 g, corn oil 1 g, cholesterol 0.25 g, sucrose 40.5 g, corn starch 10 g, cellulose 5.9 g, minerals 2.6 g, and vitamins 8.2 g (Hope Farms, Woerden, The Netherlands). This diet was supplemented with either 0.05% (w/w) cafestol and 0.025% (w/w) kahweol (high cafestol diet), 0.01% (w/w) cafestol and 0.005% (w/w) kahweol (low cafestol diet), or no cafestol and kahweol (placebo diet). Standardized to daily energy intake, these amounts are comparable with a daily amount of 40, 8 or 0 cups of unfiltered coffee, respectively, per 10 MJ (the average daily energy intake in humans). Diets were stored at -20°C until use and food was renewed twice a week. Institutional guidelines for animal care were observed in all experiments.

Experimental design

Per mice strain, animals were randomly divided into 3 experimental groups of 8 mice each, matched by age. During a run-in period of 4 weeks, all mice received the placebo diet. During the treatment period of 8 weeks, the groups consumed either the high or the lowcafestol diet, or the placebo diet. About 100 μL of blood was taken at week 0, 2, 4 and 8 of the treatment period by orbital puncture after an overnight fasting period. Feces were sampled during three days in week 3 and in week 6 of the experimental period. They were stored at -20°C until analysis of bile acids. After 8 weeks of treatment, mice were anaesthetized with ether, bled and cervical dislocated.

Measurement of serum lipids and lipoproteins

In serum, total cholesterol and triglycerides (without free glycerol) were measured enzymatically (CHOD-PAP method, Boehringer Mannheim, #236691, and GPO-trinder, Sigma, #337-B, respectively). Alanine aminotransferase was measured enzymatically (GPT, Boehringer Mannheim, #745138) after pooling the serum of 4 mice in one cage. Subsequently, serum lipoproteins were separated by ultracentrifugation. For this, 25 μL of serum per mouse was pooled per treatment group. 200 μL of pooled serum was layered with 1 mL of potassium bromide (ρ=1.21), 2.58 mL of sodium chloride (ρ=1.063), and 8 mL of distilled water in a poly-allomer tube (Beckman instruments, Mijdrecht, The Netherlands). The tubes were centrifuged for 18 h at 40.000 rpm at 4°C in a Beckman SW41 rotor. Then, the volume was fractionated in 47 fractions using a peristaltic pump (LKB Micro Perspex) and a fractionating apparatus (LKB Redifrac). In each fraction, cholesterol was measured enzymatically as described above, and the density was checked with a densitometer (Mettler DMA 45, Graz, Austria).

Enzyme activity of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

In livers from mice which had been on a high cafestol or placebo diet for 3 weeks, enzyme activities of cholesterol 7α-hydroxylase in microsomes and sterol 27-hydroxylase in mitochondria were determined essentially according to Chiang (14). This method measures the mass conversion of cholesterol into 7α- and 27-hydroxycholesterol. Briefly, 1 mg of either microsomal or mitochondrial protein was incubated in 1 mL of buffer containing 0.1 mol/L

potassium phosphate pH 7.2, 50 mmol/L NaF, 5 mmol/L DTT, 1 mmol/L EDTA, 20% (w/v) glycerol and 0.015 % (w/w) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS). Twenty µL of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated under agitation for 10 min at 37°C. Then 200 µL of a regenerating system containing 10 mmol/L sodium isocitrate, 10 mmol/L $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase was added at 37°C. After 20 min of incubation, 60 µL of a stop solution containing 20% (w/v) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. After addition of 100 µL buffer containing 0.1% (w/v) cholesteroloxidase (Calbiochem, USA, #228250), 10 mmol/L potassium phosphate pH 7.4, 1 mM DTT and 20% glycerol (w/v), steroid products were oxidized at 37 °C for 45 min. The reaction was stopped by the addition of 2 mL ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues were resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v). This mixture was analyzed using a C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 50% acetonitril and 50% (v/v) methanol at a flow rate of 0.8 mL/min. The amount of the products formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

RNA isolation, blotting and hybridization procedures

Isolation of total RNA, and subsequent electrophoresis, Northern-blotting and hybridization techniques were performed as described previously (5,15). The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region; a 1.6 kb HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA, a 1.2 kb HindIII fragment of murine oxysterol 7α-hydroxylase cDNA (16), and a 2.2 kb EcoRI fragment of rat LDL-receptor cDNA. As controls, a 1.2 kb PstI fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and a 3.8 mDa EcoRI fragment of the human 18S ribosomal DNA were used (17). The GAPDH mRNA or 18S rRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantitated using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Determination of total bile acid concentration and bile acid composition in feces

Dried feces (25 mg) were treated with 1 mL alkaline methanol (methanol : 1 mol/L NaOH 3:1 (v/v) for 2 h at 80°C in screw capped tubes. Then 9 mL of distilled water was added and the tubes were mixed and centrifuged. Total bile acid concentration was determined enzymatically/fluorimetrically on 100 µL of the supernatant applying 3α-hydroxysteroid dehydrogenase (18,19). The total residual supernatant was subsequently applied to a prepared Sep-Pak C18 solid phase extraction cartridge for determination of individual bile acid concentrations (19). After a clean up by wash procedures, bile acids were eluted with 75% methanol (20). Coprostanol was used as an internal standard. The eluate was evaporated to dryness and the bile acids were methylated with acetyl chloride/methanol 1:20 (v/v) for 30 min at 60°C. The samples were then evaporated to dryness and silylated with 100 µl bis-trimethylsilyl- trifluoroacetamide (BSTFA)/pyridine/trimethylchlorosilane (TMCS) 5:4:1

(v/v) at room temperature for at least 1h. The methyl-TMS derivatives were separated on a 25 m x 0.25 mm capillary OV-1701 GC column (CP Sil 19CB, Chrompack International, Middelburg, The Netherlands) (20) in a HP6890 gas chromatograph (Hewlett Packard, Palo Alto, Ca, USA) equipped with a flame ionization detector (FID). The injector was kept at 280°C, the FID detector at 300°C. Helium was used as carrier gas at a flow rate of 0.8 mL/min. The column temperature was programmed from 240 to 280°C at a rate of 10°/min. Bile acid derivatives were introduced by split-injection (split ratio 20:1). Quantitation was based on the area ratio of the individual bile acid to the internal standard.

Determination of neutral sterol composition and concentrations in feces
Dried feces (25 mg) were treated with 1 mL alkaline methanol as described for bile acid measurement to liberate neutral sterols from feces material. Prior to this treatment 5α-cholestane was added as internal standard. After treatment the tubes were cooled to room temperature and the neutral sterols extracted three times with 3 mL petroleum ether. The combined petroleum ether layers were evaporated to dryness and the neutral sterols silylated to TMS derivatives using the same protocol as described for bile acids. Analysis of the TMS derivatives was performed by GC applying the same column and analytical conditions as described for the methyl TMS derivatives of bile acids. Quantitation was based on the area ratio of the individual neutral sterol to the internal standard 5α-cholestane.

Measurement of liver lipids
Liver samples from mice which had been on a high cafestol or placebo diet for 3 weeks were homogenized and samples were taken for measurement of protein content. 2 µg of cholesterol acetate was added per sample as an internal standard. Then, lipids were extracted from the homogenate according to Bligh & Dyer (21). The neutral lipids were separated by high performance thin layer chromatography on silica-gel-60 pre-coated plates as described previously (5,22). Quantitation of the lipid amounts was performed by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density areas with the computer program Tina version 2.09.

***In vivo* hepatic VLDL production in apoE*3-Leiden mice**
Mice which had been on a high cafestol or placebo diet for 3 weeks were fasted for 4 h (from 8.00-12.00 a.m.) and then injected with Triton WR 1339 (500 mg/kg body weight). Triton virtually completely inhibits serum VLDL clearance (23). Subsequently, serum triglycerides were determined prior to injection (t=0 min) and 30, 60, 90, 120 and 180 min after Triton injection. The hepatic VLDL production rate was calculated from the slope of the curve and expressed as µmol/h/kg body weight. Serum collected 180 min after Triton injection was pooled per treatment group and VLDL was subsequently isolated by ultracentrifugation in triplicate. Triglycerides, total and free cholesterol, and phospholipids were measured enzymatically as described previously (24). Cholesteryl esters were subsequently calculated as the difference between total and free cholesterol.

Statistical analyses
We calculated the change in serum lipids per mouse by subtracting values at the start of the experimental period from values obtained during the experimental period. After checking for normality, differences in changes between treatment groups and the control group were

Table 1. Food intake, body weight and serum ALT in female apolipoproteinE*3-Leiden (apoE*3-Leiden), heterozygous LDL receptor knock-out (LDLr+/-) and wild type (WT) C57Bl/6 mice fed on moderate cholesterol diets enriched with either 0.05% w/w (high cafestol diet), 0.01% w/w (low cafestol diet) or no cafestol (placebo diet) for 8 weeks.

	Placebo diet	Low cafestol diet	High cafestol diet
ApoE*3-Leiden *mice*			
Food intake (g/d)	2.8±0.2	2.6±0.2†	2.3±0.2†
Body wt (g)			
Initial	20.4±2.9	20.1±2.4	19.8±0.9
Final	23.3±2.7	21.9±2.0	20.9±1.1†
ALT (U/L)			
Initial	100.3±16.5	95.8±5.9	92.3±3.2
Final	107.0±4.2	57.2±7.8	68.4±8.5
LDLr+/- mice			
Food intake (g/d)	2.6±0.2	2.4±0.1†	2.2±0.2†
Body wt (g)			
Initial	20.4±2.6	21.1±2.5	23.0±2.4
Final	22.1±2.8	22.2±2.8	21.3±1.6†
ALT (U/L)			
Initial	54.1±24.4	71.9±6.6	51.0±22.3
Final	30.9±9.5	28.3±2.8	33.8
WT mice			
Food intake (g/d)	2.7±0.1	2.5±0.1†	2.4±0.4†
Body wt (g)			
Initial	21.3±1.3	21.0±0.8	21.2±1.3
Final	23.4±0.8	22.2±1.0	20.9±1.1†
ALT (U/L)			
Initial	33.1±5.1	41.7±10.9	54.6±32.4
Final	26.3±6.0	26.5±8.6	23.0±3.3

* Mean (± SD) of eight mice per strain per diet (ALT analyses were determined in duplicate per mice strain per diet, except for the final value of ALT in LDLR+/- mice on the high cafestol diet, which was determined in one pool sample). † ($p<0.05$) compared to the placebo diet (Student's T-test on changes during the experimental period)

tested using the one-tailed unpaired Student's t-test. Other data were analyzed statistically using a two tailed Student's unpaired t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± SD.

RESULTS

Food, body weight and alanine aminotransferase

In all mice strains, the average change in body weight was significantly higher in mice fed the placebo diet compared to mice fed the high cafestol diet ($p<0.05$); average daily food intake was significantly higher in mice fed the placebo diet compared to mice fed the low and high cafestol diet ($p<0.05$). Concentrations of the liver enzyme alanine aminotransferase (ALT) did not significantly increase during consumption of cafestol in all mice strains (Table 1).

Cafestol increases serum lipid and lipoprotein level

Cafestol raised serum cholesterol in all three mice strains after 8 weeks of dietary intervention (Figure 1). In apoE*3-Leiden transgenic mice, serum cholesterol was raised by 33% (3.46 mmol/L; 95%CI [1.62;5.30]) in the low cafestol diet group and by 61% (6.35 mmol/L; 95%CI [4.47;8.22]) in the high cafestol diet group. In LDLR+/- mice, serum cholesterol was raised by 20% (0.85 mmol/L; 95%CI [-0.25;1.94]) in the low cafestol diet group and by 55% (2.37 mmol/L; 95%CI [0.73;4.01]) in the high cafestol diet group. In WT mice, serum cholesterol was raised by 24% (0.62 mmol/L; 95%CI [0.34;0.90]) in the low cafestol diet group and by 46% (1.21 mmol/L; 95%CI [0.92;1.21]) in the high cafestol diet group. In all mice strains, the rise in serum cholesterol was predominantly due to a rise in VLDL and IDL cholesterol (Figure 2). Serum triglycerides were increased after 2 weeks in apoE*3-Leiden transgenic and WT mice and remained significantly higher in the apoE*3-Leiden transgenic mice during cafestol treatment compared to placebo treatment (Figure 1).

Since the effects on serum cholesterol were most pronounced in the apoE*3-Leiden transgenic mice, we proceeded with this animal model to study the mechanism of the cholesterol-raising effects of cafestol.

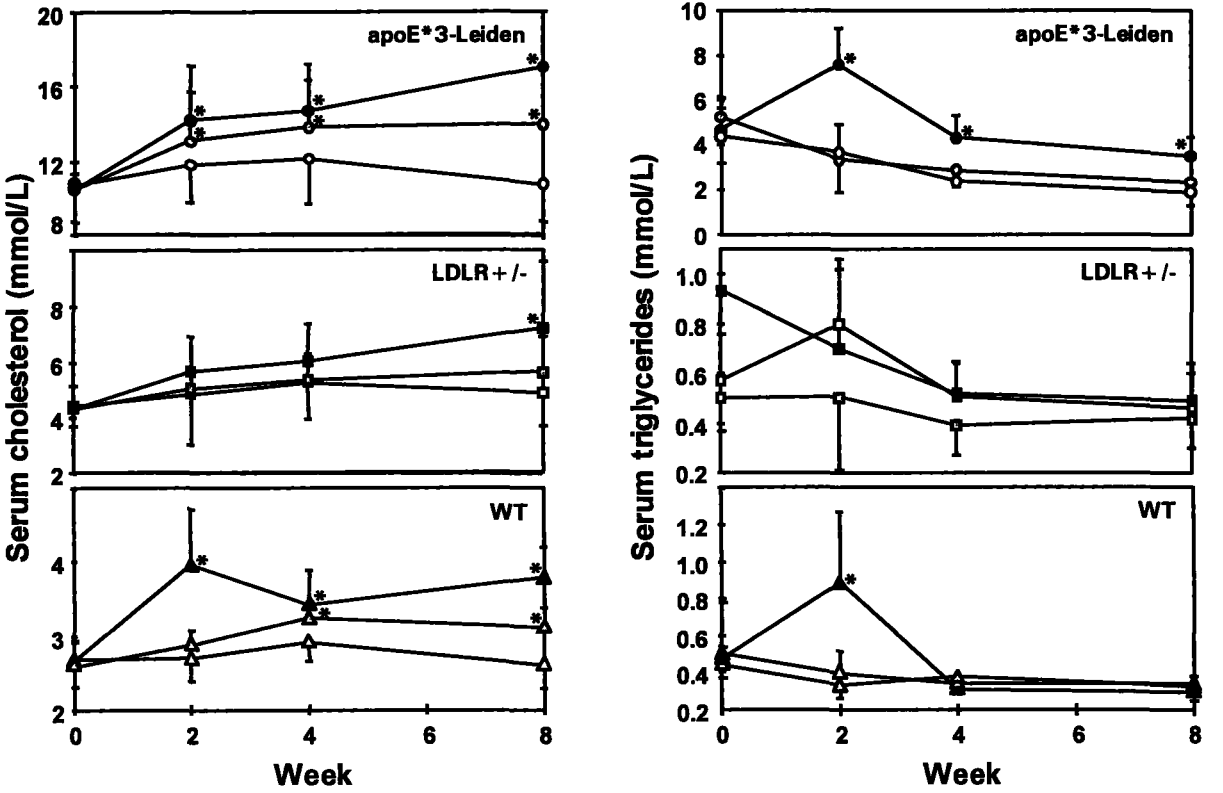

Fig. 1 Effect of a high cafestol (0.05% w/w) diet (black marks), a low cafestol (0.01% w/w) diet (gray marks) and a placebo diet (white marks) on serum cholesterol and triglycerides in apoE*3-Leiden mice (circles), LDLR+/- mice (squares) and WT (C57Bl/6) mice (triangles). Significant differences between the cafestol and the placebo treatment are indicated by an asterisk*.

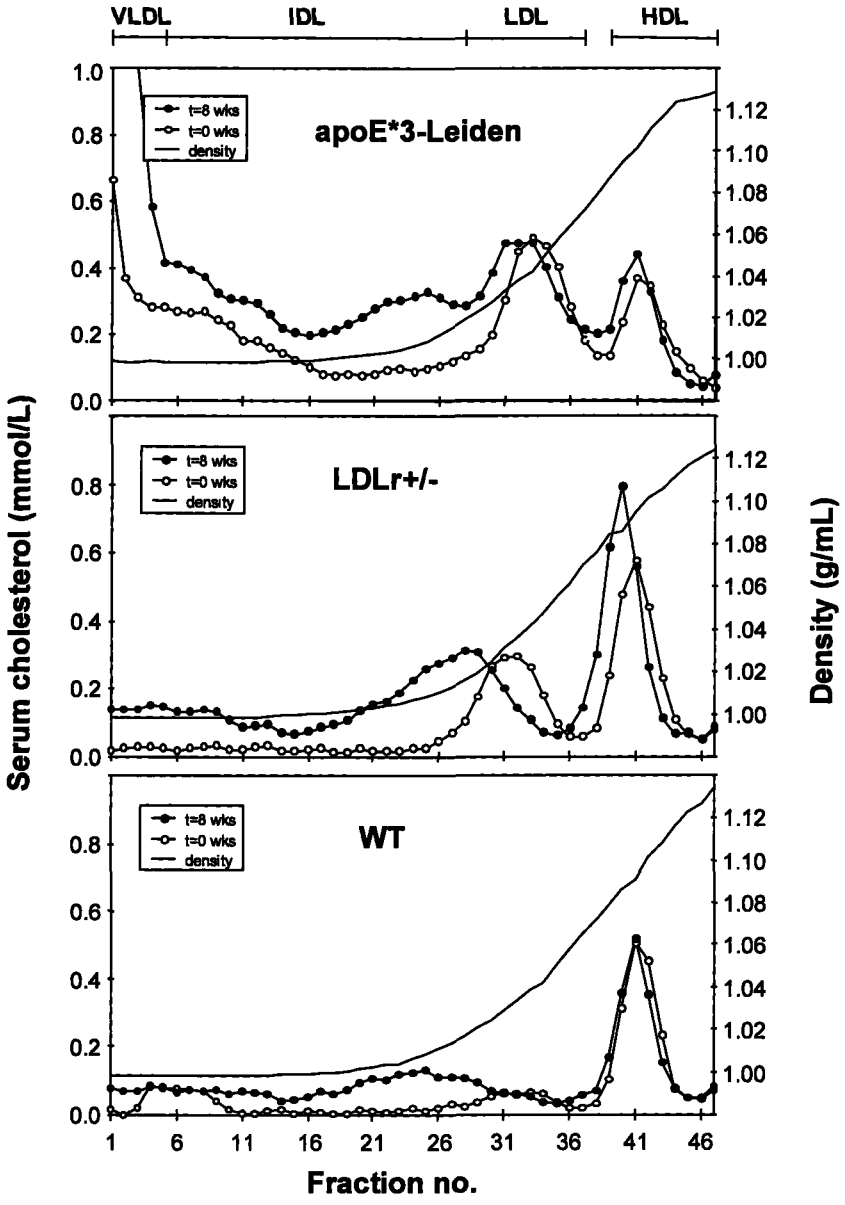

Fig 2. Effect of a high cafestol (0.05% w/w) diet on cholesterol profiles in apolipoprotein E*3-Leiden mice (upper part), LDLR+/- mice (middle part) and WT (C57Bl/6) mice (lower part). The white marks indicate the cholesterol profile from pooled serum of 8 mice at the start of the experimental period (t=0). The black marks indicate the cholesterol profile from pooled serum of 8 mice after 8 weeks of treatment with the high cafestol diet. Serum was fractionated as described in Animals and Methods.

Cafestol decreases hepatic enzymes in bile acid synthesis and fecal excretion of bile acids

To validate the effects of cafestol on bile acids synthesis obtained in cultured rat hepatocytes (5), we determined the effect of a high cafestol diet during 3 weeks on enzymes involved in bile acid synthesis and on fecal bile acid excretion in apoE*3-Leiden transgenic mice. Cafestol decreased the cholesterol 7α-hydroxylase activity and mRNA levels by 57% and 58%, respectively (both $p<0.05$) (Table 2). Cafestol also decreased sterol 27-hydroxylase mRNA levels by 32% ($p<0.05$), while the enzyme activity was paradoxically increased by 40% ($p<0.05$) (Table 2). It is well-known, however, that mitochondria may contain oxysterol 7α-hydroxylase (25), as found in pigs (26) and in humans (27) converting 27-hydroxycholesterol into 7α,27-dihydroxycholesterol (25-27). Thus, it is possible that the apparent increase in sterol

Table 2. Effect of cafestol on hepatic mRNA and activity levels in apo E3-Leiden mice

	Placebo diet		High cafestol diet	
	activity	mRNA	activity	mRNA
	(% of placebo)			
Cholesterol 7α-hydroxylase	100 ± 5	100 ± 41	43 ± 1*	42 ± 7*
Sterol 27-hydroxylase	100 ± 10	100 ± 12	140 ± 5*	68 ± 17*
Oxysterol 7α-hydroxylase	N.D.	100 ± 21	N.D.	41 ± 16**
LDLR	N.D.	100 ± 26	N.D.	63 ± 14*

ApoE*3-Leiden mice were treated with a high cafestol diet or a placebo diet for 3 weeks. Hepatic enzyme activities and mRNA levels of mice treated with a high cafestol (0.05% w/w) or placebo diet were determined after a 4 hour fasting period from 8 till 12 a.m. The amount of mRNA was corrected for differences in total RNA applied to the gel, using 18 S ribosomal RNA as an internal standard. Absolute activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase from apoE*3-Leiden mice treated the placebo diet were 1.83 nmol/h/mg protein and 1.76 nmol/h/mg protein, respectively. Data shown are means ± SD (n=4 per group). A significant difference between placebo and cafestol diet is indicated by an asterisk* (*$p<0.05$*), **(*$p<0.005$*). N.D.: not determined.

27-hydroxylase activity can be attributed to accumulation of its product, 27-hydroxycholesterol, caused by a blockade in the subsequent metabolic conversion. Therefore, we measured mRNA levels of oxysterol 7α-hydroxylase. The expression of this enzyme was decreased by 58% ($p<0.005$), giving an explanation for the apparent increase in sterol 27-hydroxylase activity (Table 2).

Since down-regulation of enzymes involved in bile acid synthesis has consequences for the overall process of bile acid production, the amount of total and individual bile acids was measured in feces. Cafestol decreased the total amount of bile acids excreted in the feces with 41% as compared to the placebo diet (Table 3). In addition, cafestol changed the relative amount of the various bile acids in the feces showing an increase in the ratio between bile acids formed only via the neutral or 7α-hydroxylated pathway (deoxycholate and cholate) and those formed via the neutral as well as the acidic or 27-hydroxylated pathway (28,29) (remaining bile acids) (Table 3). Furthermore, the amount of fecal neutral sterols excreted in the cafestol treated group was slightly lower as compared to the placebo group indicating that inhibition of bile acid synthesis does not lead to an increased secretion of free cholesterol into bile (Table 3). The effect on the excretion and composition of fecal bile acids and neutral sterols was similar after 3 and 6 weeks on the high cafestol or placebo diet.

Effect of cafestol on VLDL production and hepatic lipid metabolism in apoE*3-Leiden transgenic mice

In order to investigate the effects of a decreased bile acid synthesis on hepatic lipid metabolism, we determined the amount of hepatic lipids of apoE3*Leiden transgenic mice treated with a high cafestol or placebo diet for 3 weeks. Cafestol decreased the hepatic triglyceride content by 52% ($p<0.05$), but it did not alter the liver free and esterified cholesterol levels (Table 4). Although the hepatic content of free cholesterol in apoE*3-Leiden mice was apparently not affected by cafestol, the regulatory pool of cholesterol was increased as indicated by a decreased expression of the LDL-receptor (-37%, $p<0.05$) (Table 2).

Since the excess hepatic cholesterol was not excreted into the bile, it might have been secreted by the liver in VLDL particles. Therefore, we measured nascent VLDL production in apolipoprotein E*3-Leiden transgenic mice after 3 weeks on a high cafestol or placebo diet. The relative amount of cholesteryl esters in the VLDL particles upon cafestol treatment was almost 3 times higher compared to placebo treatment ($p<0.05$), while the relative amount of triglycerides in the particles was

Table 3. Effects of cafestol on fecal bile acid excretion and composition and on excretion of neutral sterols of apo E3-Leiden mice

	Bile acid excretion	DC	C	LC	CDC	HDC	UDC	α/βMC	ωMC	ratio (7α/7α+27)	Neutral sterol excretion
Treatment	µmol/day per 100g bw	(%)								(%)	µmol/day per 100g bw
Placebo diet	6.8 ± 0.7	21	15	4	1	2	3	28	26	36/64	34.3 ± 1.3
	(100%)										(100%)
High cafestol diet	4.0 ± 0.1	30	13	3	1	2	3	16	32	43/57	27.9 ± 2.8
	(59%)										(81%)

Mice were treated with a high cafestol diet or a placebo diet for 3 weeks. In feces, total bile acids, neutral sterols and bile acid composition was determined as described in "Methods". Data are means ± range of 2 individual feces samples of each group. DC: deoxycholate; C: cholate; LC: lithocholate; CDC: chenodeoxycholate; HDC: hyodeoxycholate; UDC: ursodeoxycholate; MC: muricholate. Ratio 7α/7α+27 indicates Σ(DC+C)/Σ(LC+CDC+HDC+UDC+all MC's). Values between parenthesis represent the percentage of the value obtained in animals treated with the placebo diet.

Table 4. Effect of cafestol on hepatic cholesterol and triglyceride content in apo E3-Leiden mice

	FC	CE	TG
Treatment		(µg/mg protein)	
Placebo	15.5 ± 1.5	38.3 ± 7.7	86.3 ± 10.6
Cafestol	14.4 ± 1.3	32.8 ± 11.9	41.1 ± 8.7*
	(93%)	(85%)	(48%)

Mice were treated with a high cafestol diet or a placebo diet for 3 weeks. In liver homogenates, free cholesterol (FC), cholesteryl esters (CE) and triglycerides (TG) were determined using a h.p.t.l.c. method (see "Methods") after a 4 hour fasting period. Data are means ± SD (n=4). Values between parenthesis represent the percentage of the value obtained in animals treated with the placebo diet. A significant difference between control and treated mice is indicated by an asterisk * ($p<0.05$).

Table 5. Effect of cafestol on VLDL composition in apo E3-Leiden mice.

	TG	FC	CE	PL
Treatment		% of total lipid (by weight)		
Placebo	52.0 ± 3.0	7.8 ± 0.8	17.5 ± 2.9	22.7 ± 5.0
Cafestol	28.9 ± 10.1*	6.6 ± 1.1	49.2 ± 12.7*	15.3 ± 1.7

VLDL was isolated by ultracentrifugation from pooled serum of 8 fasted apo E*3-Leiden transgenic mice treated with a high cafestol or a placebo diet for 3 weeks. The serum was collected 180 min after Triton injection. Triglycerides (TG), total cholesterol, free cholesterol (FC) and phospholipids (PL) were measured enzymatically and the amount of cholesteryl ester (CE) was calculated (see "Methods") Data are means ± SD of three individual VLDL samples of each group. A significant difference between control and cafestol treated mice is indicated by an asterisk * ($p<0.05$).

decreased by about 50% (Table 5). This was reflected in a two times lower VLDL triglyceride production rate (35.1±13.8 µmol/h/kg after the high cafestol diet versus 63.1±17.5 µmol/h/kg after the placebo diet) (Figure 3).

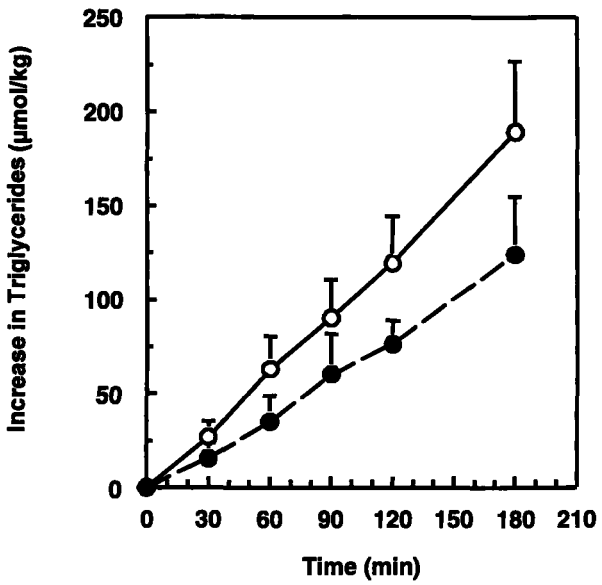

Fig 3. Effect of cafestol on the VLDL-triglyceride production rate in apoE*3-Leiden mice.
Triton WR 1339 (500 mg/kg body weight) was injected into placebo (white marks) and cafestol (0.05% w/w) (black marks) treated apoE*3-Leiden mice (n=8 fasted mice per group). Serum triglyceride levels were determined at 30, 60, 90, 120 and 180 min and corrected for the triglyceride level at the time of injection (t=0 min) The values shown are means ± SD.

DISCUSSION

In this study, cafestol increased serum cholesterol levels in apoE*3-Leiden, heterozygous LDL receptor deficient, and in wild type (C57Bl/6) mice, mainly in the VLDL and IDL fraction. In apoE*3-Leiden transgenic mice, cafestol decreased bile acid synthesis reflected by a reduction in the total amount of fecal bile acids by down-regulation of expression of enzymes involved in the neutral as well as in the alternative bile acid synthetic pathway. The decrease in bile acid synthesis, resulted in a decline in LDL receptor mRNA levels and an increased secretion of VLDL cholesterol ester.

So far, no suitable animal model has been found to study the mechanism of action of cafestol, since various animal models did not respond to this coffee diterpene as humans do (6-10). These mice are the first animals showing a similar increase in serum cholesterol due to cafestol as observed in humans, making this species a good model to investigate the biochemical background of the cholesterol-raising effect of cafestol in humans. It should be noted that the increase in serum cholesterol in humans is mainly present in LDL, whereas in the mice the rise is predominantly found in the VLDL-IDL range.

Previously, we reported that cafestol suppressed bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression in rat

hepatocytes (5). This is now confirmed *in vivo* in apolipoprotein E*3-Leiden transgenic mice. In this study we also showed that the total bile acid mass in feces of the cafestol- treated group was decreased. Cafestol predominantly affected cholesterol 7α-hydroxylase and oxysterol 7α-hydroxylase, but the effects on sterol 27-hydroxylase also contributed to the decreased fecal bile acid mass since cafestol changed the fecal bile acid composition. The ratio between bile acids formed only via the neutral or 7α-hydroxylated pathway (deoxycholate and cholate) and those formed via the neutral as well as the acidic or 27-hydroxylated pathway (remaining bile acids) (28,29) increased. This indicates inhibition of the acidic pathway in bile acid synthesis next to an inhibitory effect on the neutral pathway.

Theoretically, a suppressed bile acid synthesis would increase the pool of free cholesterol in the liver cell. We did not find an effect on hepatic free cholesterol levels, but different metabolic pathways might have converted the free cholesterol into cholesteryl esters or removed it from the liver as such and/or via VLDL particles to maintain the hepatic cholesterol homeostasis. Since we did not find an increase in fecal excretion of neutral sterols or a hepatic accumulation of cholesteryl esters, it appears plausible that the cholesterol which becomes available due to inhibition of bile acid synthesis is directly removed from the liver via VLDL particles (see below). In addition, high amounts of free cholesterol in the cell membranes (30) may overshadow subtle changes in free cholesterol caused by inhibition of bile acid synthesis. We found a substantial decrease in LDLR mRNA, which is a sensitive measure to detect changes in the regulatory pool of free cholesterol. Subtle increases in intracellular cholesterol prevent processing of sterol regulatory element binding protein (SREBP), resulting in a down-regulation of LDL-receptor gene transcription (31). A similar decrease in LDLR mRNA levels has been shown *in vitro* in cultured rat hepatocytes (5) and in HepG2 cells (32). In contrast, divergent data were reported in other cell types (33,34), possibly because of the different metabolic functions of these cells. Our results plead in favor of the hypothesis that the cholesterol-raising effect of cafestol can be explained at least in part by a reduced expression of the LDL receptor.

The rise in serum cholesterol upon cafestol treatment may also partly be explained by an increased secretion of cholesteryl esters in VLDL. The relative amount of cholesteryl esters in the VLDL particles upon cafestol treatment was significantly higher compared to placebo treatment. Concomitantly, the relative amount of triglycerides in the particles decreased. This reduction was to the same extent as the decline in VLDL-triglyceride production rate. This suggests that not the

number of particles but the composition is changed, resulting in the production of a ßVLDL-like particle. The decrease in VLDL-triglyceride production rate and the reduced triglyceride content of the liver suggests an impaired triglyceride synthesis. Whether this is due to a direct or indirect effect of cafestol on activity or expression of enzymes involved in triglyceride synthesis awaits further investigation.

In conclusion, we found that cafestol inhibits bile acid synthesis by down-regulation of both the neutral and the acidic pathway leading to a decrease in the expression of the LDL receptor and an elevated secretion of cholesteryl esters in VLDL. Suppression of bile acid synthesis may provide an explanation for the cholesterol-raising effects of unfiltered coffee in humans.

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Chapter 6

Fibrates suppress bile acid synthesis via PPARα-mediated down-regulation of cholesterol 7α-hydroxylase gene expression

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Submitted

Fibrates suppress bile acid synthesis via PPARα-mediated down-regulation of cholesterol 7α-hydroxylase gene expression

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Running title: Fibrates inhibit bile acid synthesis

ABSTRACT

Fibrates are widely used hypolipidemic drugs which activate nuclear peroxisome proliferator-activated receptors (PPARs) and thereby affect the expression of different genes involved in lipid metabolism. Treatment with these drugs causes adverse changes in the biliary lipid composition and decreases the excretion of bile acids leading to an increased incidence of cholesterol gallstones. We studied the mechanism of regulation of bile acid synthesis and cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene expression by fibrates in cultured hepatocytes and *in vivo* in rats and mice.

Ciprofibrate (300 μM) and the PPARα agonist Wy14,643 (100 μM) decreased bile acid synthesis in rat hepatocytes (-66% and -61%). Ciprofibrate and Wy14,643 suppressed activities of cholesterol 7α-hydroxylase (-69% and -60%, respectively) and sterol 27-hydroxylase (both -49%), paralleled by a similar reduction of the respective mRNAs. Treatment of rats with 0.05% (w/w) ciprofibrate decreased cholesterol 7α-hydroxylase enzyme activity (-87%) and mRNA levels (-69%).

Evidence for the functional involvement of PPARα in the suppression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase *in vivo* was obtained using PPARα null (-/-) mice. In wild-type mice, ciprofibrate reduced cholesterol 7α-hydroxylase (-65%) and sterol 27-hydroxylase mRNA levels (-48%). However, in PPARα -/- mice this effect was completely abolished. Promoter-reporter studies

showed the presence of a functional PPAR-responsive element in the proximal promoter of the cholesterol 7α-hydroxylase gene and/or interference with HNF-4-mediated activation of cholesterol 7α-hydroxylase gene, which both can mediate suppression by fibrates.

A decreased production of bile acids by PPARα-mediated down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase may contribute to the increased risk for gallstone formation in patients treated with fibrates.

INTRODUCTION

Fibrates are drugs which are widely used in the treatment of hyperlipidemia (1). The drugs lower triglyceride levels and increase HDL-cholesterol in hyperlipidemic patients (2-4).

Fibrates act by activation of specific nuclear receptors, termed peroxisome proliferator-activated receptors (PPARs). After heterodimerization with the retinoid X receptor (RXR), they alter the transcription of specific genes controling lipoprotein metabolism by binding to a distinct response-element, the peroxisome proliferator response element (PPRE). The PPRE consists of a direct repeat of the AGGTCA consensus sequence separated by 1 or 2 nucleotides (DR-1, DR-2) (1,5,6). Three different types of PPARs have been identified in different species, termed α, δ (also called ß) and γ, each encoded by a separate gene and showing a distinct distribution pattern (7). PPARα is highly expressed in the liver and its activation results in an increased fatty acid catabolism (1).

It has been established that fibrate treatment causes adverse changes in the biliary lipid composition and decreases the excretion of bile acids leading to supersaturation of gall bladder bile and consequently to an increased incidence of cholesterol gallstones in patients undergoing long-term therapy (8-12). Bile acid synthesis and secretion in combination with the excretion of free cholesterol into the bile is the major route for the elimination of cholesterol from the mammalian body (13,14). The classical or neutral route in bile acid biosynthesis in rats and humans is initiated by 7α-hydroxylation of cholesterol catalyzed by the major rate-limiting enzyme cholesterol 7α-hydroxylase, which is located in the smooth endoplasmic reticulum (15,16). An alternative pathway in bile acid synthesis is operational as well, contributing considerably to the total bile acid synthesis in humans (17), rats (18),

rabbits (19) and in cultured human and rat hepatocytes (20). The latter so-called acidic pathway is initiated by the conversion of cholesterol by the enzyme sterol 27-hydroxylase, which is located in the inner mitochondrial membrane (15,16,21). A decreased enzyme activity of cholesterol 7α-hydroxylase was found in humans treated with fibrates (11,22). However, the mechanism of action and the potential role of PPARα in this process remains to be clarified. Furthermore, the effect of fibrates on sterol 27-hydroxylase expression has not been established.

In this study we further examined the effect of fibrates in the regulation of bile acid synthesis and more specifically on the expression of the major enzymes involved in this process, cholesterol 7α-hydroxylase and sterol 27-hydroxylase, using cultured rat hepatocytes and *in vivo* in rat. To establish the functional role of PPARα in the regulation of bile acid synthesis we studied the effects of fibrates in PPARα-null (-/-) mice. Promoter-reporter studies were applied to delimitate a functional PPRE in the proximal cholesterol 7α-hydroxylase promoter.

Our data indicate that fibrates suppress bile acid biosynthesis in rodents via PPARα-mediated down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene expression.

MATERIAL AND METHODS

Material and animals

Ciprofibrate, fenofibric acid and gemfibrozil were kindly provided by Dr. M. Riteco (Sanofi Winthrop, Maassluis, The Netherlands), Dr. A. Edgar (Laboratoires Fournier, Daix, France), Dr. B. Bierman (Warner-Lambert, Hoofddorp, The Netherlands), respectively. Bezafibrate was obtained from Boehringer Mannheim (Almere, The Netherlands). BRL49653 was a gift from Dr. Berthelon (Lipha Merck, Lyons, France). Materials used for the isolation and culturing of rat hepatocytes, and assaying cholesterol 7α-hydroxylase and sterol 27-hydroxylase were obtained from sources described previously (23-26). [α-^{32}P]dCTP (3000 Ci/mmol) was obtained from Amersham Life Sciences, Buckinghamshire, UK.

Male Wistar rats weighing 250-350 g were used throughout and were maintained on standard chow and water *ad libitum*. *In vitro* experiments: Two days before the isolation of hepatocytes, the rats were fed a diet supplemented with 2% (w/w) cholestyramine (Questran, Bristol Myers B.V. Weesp, The Netherlands). For the preparation of hepatocytes, the animals were killed between 9 and 10 a.m. *In vivo* experiments: Male Wistar rats were divided in groups of four animals each and treated for 14 days with ciprofibrate 0.05% (w/w) in standard rat chow. At the end of the treatment period rats were fasted overnight. Male Sv/129 homozygous wild-type (+/+) and PPARα-null (-/-) mice, which were kindly provided by Dr. F. Gonzalez (27) (10-12 weeks of age) were fed for 17 days with standard mice chow mixed with 0.05% (w/w) ciprofibrate. At the end of the treatment period, the animals were fasted for

4 hours, weighed and sacrificed by exsanguination under ether anaesthesia. For isolation of RNA, microsomes and mitochondria livers were removed immediately, weighed, rinsed with 0.9% (w/v) NaCl and frozen in liquid nitrogen. None of the treatments caused changes in the amount of food consumed by the animals. Institutional guidelines for animal care were observed in all experiments.

Rat hepatocyte isolation and culture

Hepatocytes were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor and cultured as described previously (23,24,26). After a 4-hour attachment period, the cell medium was refreshed with 1.0 ml (6-well plates) or 2.5 ml (dishes) of Williams E (WE) medium supplemented with 10% fetal calf serum (FCS), and cells were incubated for a further 14 hours. Various fibrates and Wy14,635, dissolved in DMSO, were added to the culture medium, at between 18 and 42 hours of culture age, unless otherwise stated. The final concentration of DMSO in the medium was 0.1% (v/v). Cells were harvested at the same time after a 42-hour culture period for measurement of cellular lipids, cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity, and determination of mRNA levels. Cell viability, after culturing with ciprofibrate or WY14,635, was assessed by ATP measurements and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl bromide (MTT) assays as described previously (26). The latter assay depends on the cellular reduction of MTT (Sigma Chemical Co., St. Louis, MO, USA) by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically.

Quantification of mass production of bile acids

Mass production of bile acids by rat hepatocytes was measured by gas-liquid-chromatography (g.l.c.) after a preincubation period of 8 hours (from 18-26 hours of culture age), during the following 24-hour culture period from 26-50 hours in WE medium containing 10% FCS in the presence or absence of ciprofibrate or Wy14,643 as described previously (26).

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Enzyme activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cell homogenates and isolated liver microsomes and mitochondria were determined essentially according to Chiang (28), measuring mass conversion of cholesterol into 7α- and 27-hydroxycholesterol, respectively. In short, 4 mg of protein of cell homogenates or 1 mg of either microsomal or mitochondrial protein was incubated in 1 ml of buffer containing 0.1 M potassium phosphate pH 7.2, 50 mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v) and 0.015 % (w/w) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (CHAPS). Twenty µl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated under agitation for 10 min at 37 °C. Then 200 µl of a regenerating system was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min of incubation 60 µl of a stop solution containing 20% (w/v) sodium cholate and 1 µg 20α-hydroxycholesterol, which served as a recovery standard, were added. Steroid products were oxidized at 37 °C for 45 min after the addition of 100 µL buffer containing 0.1% cholesteroloxidase (w/v) (Calbiochem, USA, #228250), 10 mM potassium phosphate pH 7.4, 1 mM DTT and 20% glycerol (w/v), and the

reaction was stopped by the addition of 2 ml ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleum ether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 50% acetonitril and 50% methanol at a flow rate of 0.8 ml/min. The amount of the product formed was determined by monitoring the absorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, Cambridge, UK).

RNA isolation, blotting and hybridization procedures

Isolation of total RNA, and subsequent electrophoresis, Northern-blotting and hybridization techniques were performed as described previously (24,26). The following DNA fragments were used as probes in hybridization experiments: a 1.6 kb PCR-synthesized fragment of rat cholesterol 7α-hydroxylase cDNA, spanning the entire coding region and a 1.6 kb *HindIII/XbaI fragment of rat sterol 27-hydroxylase cDNA. As controls, a 1.2 kb PstI fragment* of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and a 3.8 kb EcoRI fragment of the human 18S ribosomal DNA (29) were used. Either the GAPDHmRNA or 18S ribosomal RNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel or filter. mRNA levels were quantified using a Phosphor-imager BAS-reader (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.09.

Transfection experiments

Plasmid construction: The cholesterol 7α-hydroxylase (*CYP7A1*) promoter/Luciferase fusion gene pGL3-376Luc and its mutants m11 and m13 were made by subcloning the rat *CYP7A1* promoter region spanning nt -376 to nt + 32 (30) into the pGL3-Basic vector (Promega, Madison WI).

HepG2 cultures and transfection assays: The human hepatoblastoma cell line (ATCC, HB8065) was grown in DMEM/F12 (Life Technologies, Gaithersburg NJ) containing 10% (v/v) heat inactivated FCS, 100 IU/ml penicillin G and 100 µg/ml streptomycin sulfate (Life Technologies) as previously described (31). Transfection assays were performed in 48-well cluster plates by the calcium phosphate-DNA coprecipitation technique (31). Each well received 750 ng of reporter plasmid, and the indicated combinations of the expression plasmids for pCMV5-mPPARα (32) and pCMV-HNF-4 (250 ng each). For normalizing variations in transfection efficiencies 50 ng of pCMVß, a ß-galactosidase expression vector was added. Transfected cells were treated with the concentrations indicated of the PPARα agonist Wy14,643 and ciprofibrate or an equal amount of vehicle (0.1% v/v DMSO). After incubating the cultures for 20 hours in a humidified atmosphere of 5% CO_2/95% air at 37°C, cell lysates were prepared with Passive Lysis Buffer (Promega). Luciferase and ß-galactosidase assays were carried out as described (31). Each experiment was repeated at least twice with triplicate samples.

Statistical analysis

Data were analyzed statistically using Student's paired (hepatocytes) or unpaired (rats and mice) t-test with the level of significance selected to be $p<0.05$. Values are expressed as means ± S.D.

RESULTS

Ciprofibrate and Wy14,643 decrease bile acid mass production in cultured rat hepatocytes

Incubation of hepatocytes with 300 µM ciprofibrate resulted in a 66% reduction in bile acid mass production (Fig. 1). Since fibrates are PPARα activators, we tested the effect of the specific PPARα ligand Wy14,643. Incubation with 100 µM of this compound resulted in a similar decrease (-61%) in bile acid mass production, as compared to ciprofibrate (Fig. 1). The major bile acids formed were cholic acid and ß-muricholic acid, in a ratio of approximately 20:80. This ratio did not change upon incubation with both compounds. The concentrations used in these experiments (up to 1000 µM ciprofibrate and 100 µM Wy14,634) did not have adverse effects on cell viability as indicated by measurements of cellular MTT and ATP levels (103 ± 8% and 124 ± 31%, respectively for ciprofibrate; 95 ± 6% and 102 ± 10%, respectively for Wy14,643). The latter data are expressed as a percentage of control and are means ± S.D. of independent experiments using hepatocytes from 3-4 rats.

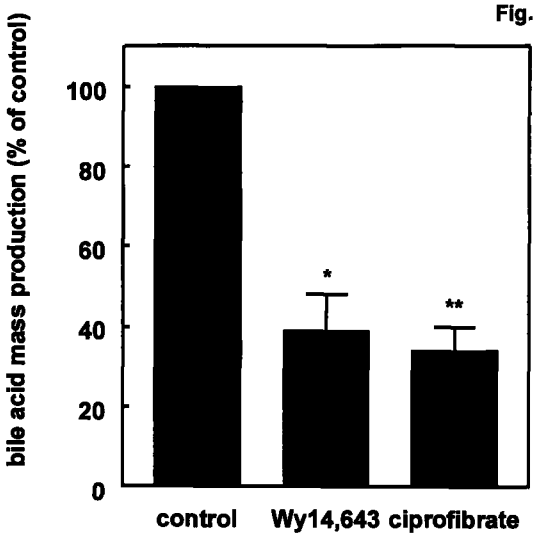

Fig. 1 Effect of ciprofibrate and Wy14,643 on mass production of bile acids.
Mass production of bile acids by rat hepatocytes was measured after an 8-hour preincubation period (from 18-26 hours of culture), during the following 24-hour culture period from 26-50 hours in WE medium containing 10% FCS in the presence or absence of 300 µM ciprofibrate or 100 µM Wy14,643. Values shown are expressed as a percentage of bile acid synthesis in control incubations and are means ± S.D. of independent experiments with hepatocytes from 3 rats. Absolute synthesis rate in control cells was 4.7 ± 1.8 µg/24hours/mg cell protein. A significant difference between control and treated cells is indicated by an asterisk (* $P<0.01$;** $P<0.005$).

Fibrates and Wy14,643 suppress cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity and mRNA levels in cultured rat hepatocytes.

To assess the level at which ciprofibrate and Wy14,643 decrease bile acid mass production, enzyme activities and mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase were determined. Ciprofibrate and Wy14,643 suppressed the

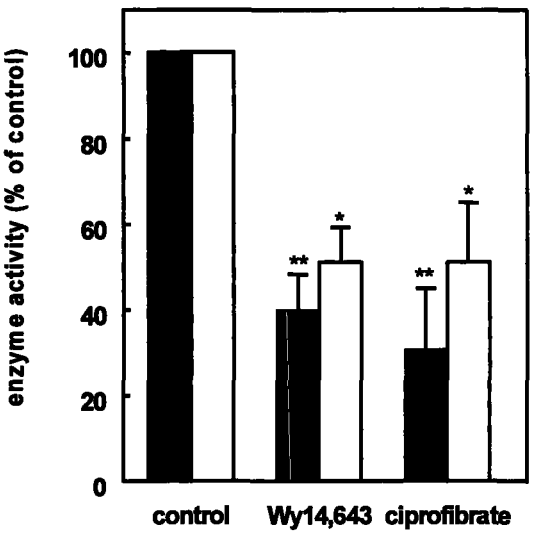

Fig. 2 Effect of ciprofibrate and Wy14,643 on cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity
Rat hepatocytes were incubated for 24 hours from 18-42 hours of culture, in the presence or absence of 300 μM ciprofibrate or 100 μM Wy14,643, and harvested for measurement of cholesterol 7α-hydroxylase (closed bars) and sterol 27-hydroxylase activity (open bars). Values shown are expressed as a percentage of enzyme activity in control cells and are means ± S.D. of independent experiments with hepatocytes from 3 to 6 rats. Absolute activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in cell homogenates in control cells were 584 ± 194 pmol/h/mg cell protein and 80 ± 7 pmol/h/mg cell protein, respectively. A significant difference between control and treated cells is indicated by an asterisk (* $P<0.05$;** $P<0.005$).

activity of cholesterol 7α-hydroxylase (-69% and -60%, respectively) and sterol 27-hydroxylase (both -49%) (Fig. 2). The decrease of cholesterol 7α-hydroxylase and sterol 27-hydroxylase activity by ciprofibrate and Wy14,643 paralleled well with the suppression of its mRNA, being -62% and -52%, respectively, for 300 μM ciprofibrate and -53% and –51%, respectively for 100 μM Wy14,643 (Table 1). Cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA levels were dose-dependently suppressed by ciprofibrate (Fig. 3). In contrast mRNA levels of GAPDH and 18S ribosomal RNA, used as internal standards, were not affected. The reduction of the bile acid synthetic enzymes in cultured rat hepatocytes by fibrates and Wy14,643 is indicative of a direct effect of these compounds on the hepatocyte.

To investigate whether the observed down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, is a general characteristic of fibrates rather than a specific effect of ciprofibrate we also studied the effect of other fibrates on mRNA levels of these enzymes (Table 1). Among the compounds tested gemfibrozil appeared to be less active in the suppression of mRNA levels of both enzymes.
In addition to their PPARα-activating capacity, fibrates also activate PPARγ, although much more weakly (33). Therefore, we tested the effect of a high affinity ligand for PPARγ, the thiazolidinedione, BRL49653. BRL49653 (10 μM) was unable to suppress cholesterol 7α-hydroxylase (105 ± 21% (n=4)) and sterol 27-hydroxylase

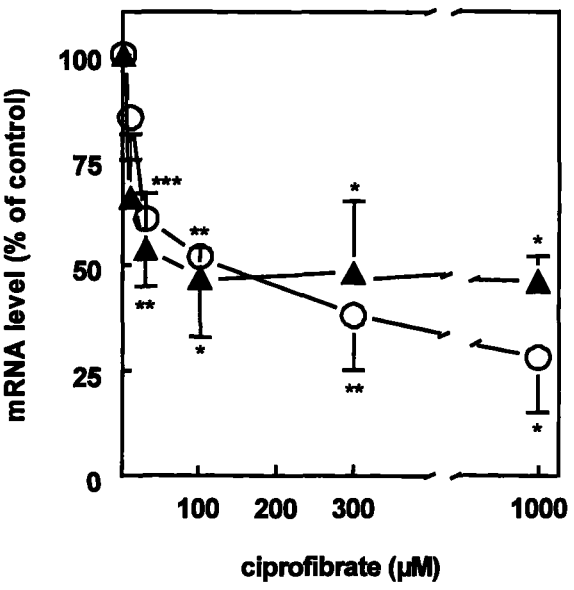

Fig. 3 Dose-dependent down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA levels by ciprofibrate.
Rat hepatocytes were incubated for 24 hours from 18-42 hours of culture, in the presence or absence of different concentrations of ciprofibrate. Cells were harvested after 24 hours of incubation to measure cholesterol 7α-hydroxylase (circles) and sterol 27-hydroxylase (triangles) mRNA levels. Values shown are expressed as a percentage of mRNA levels in control cells and are means ± S.D. of independent experiments with hepatocytes from 3-4 rats. The amount of mRNA was corrected for differences in total RNA applied to the gel, using 18S ribosomal RNA as an internal standard. A significant difference is indicated by an asterisk ($^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$).

Table 1. Effect of different clinically used fibrates and the PPARα ligand Wy14,643 on mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase

	cholesterol 7α-hydroxylase (% of control)		sterol 27-hydroxylase (% of control)	
Control	100		100	
Ciprofibrate (300 μM)	38 ± 13**	(4)	48 ± 17*	(4)
Gemfibrozil (300 μM)	90 ± 25	(4)	71 ± 10*	(4)
Bezafibrate (300 μM)	36 ±15*	(3)	43 ± 7**	(3)
Fenofibric Acid (300 μM)	73 ± 10*	(4)	59 ± 4***	(4)
Wy14,643 (100 μM)	47 ± 20**	(6)	49 ± 23**	(6)

Rat hepatocytes were incubated in the presence or absence of different fibrates or Wy14,643 for 24 hours, from 18-42 hours of culture time. mRNA levels were assessed by Northern-blot hybridization and scanning of the resulting phosphor-imager plates, using 18S ribosomal RNA as the internal standard to correct for differences in the amount of RNA applied, as described in "Material and Methods". Data are expressed as a percentage of control and are means ± S.D. of independent experiments using hepatocytes from 3-6 rats (indicated between parenthesis). A significant difference between control and treated cells is indicated by asterisks. (*, P<0.05 **,P<0.01 ***,P<0.001).

(97 ± 25% (n=4)) mRNA levels. This indicates that the suppressive effect of fibrates on cholesterol and sterol 27-hydroxylase requires PPARα and not PPARγ activation.

Ciprofibrate suppresses cholesterol 7α-hydroxylase enzyme activity and mRNA levels *in vivo* in rat

To validate the effects of fibrates obtained in cultured rat hepatocytes, we determined the effect of ciprofibrate on the major enzymes involved in bile acid synthesis *in vivo* in rat. Ciprofibrate (0.05% w/w) decreased enzyme activity and mRNA levels of cholesterol 7α-hydroxylase by 87% *($p<0.005$)* and 69% *($p<0.05$)*, respectively. In contrast to the results *in vitro* in rat hepatocytes, we did not detect significant effects on sterol 27-hydroxylase.

PPARα-/- mice are refractory to the suppressive effects of ciprofibrate on cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression

The effect of fibrates and the PPARα ligand Wy14,643 on cholesterol 7α-hydroxylase and sterol 27-hydroxylase implies a role for PPARα in the regulation of gene expression of both enzymes. To assess the direct involvement of PPARα in the regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, wild-type and PPARα-/- mice were treated with 0.05% (w/w) ciprofibrate or a control diet. Cholesterol 7α-hydroxylase mRNA levels in the wild-type mice were about 3-fold higher than in the PPARα-/- mice, whereas no significant difference in basal expression of sterol 27-hydroxylase mRNA was found between the two strains. This suggests that PPARα plays an important role in basal expression of cholesterol 7α-hydroxylase and not sterol 27-hydroxylase. In wild-type mice, ciprofibrate reduced cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA levels by 65% and 48%, respectively. In contrast, in PPARα-/- mice this effect was completely abolished (Fig 4). These results indicate that PPARα is involved in maintaining a high basal level of cholesterol 7α -hydroxylase expression and that the fibrate-suppressed expression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in wild-type mice is dependent on PPARα activation.

Site-directed mutagenesis reveals a functional PPRE in the proximal promoter of the cholesterol 7α-hydroxylase gene

Different studies (30,31,34-38) have described a region in the proximal promoter of the cholesterol 7α-hydroxylase gene, harboring major cis-acting elements,

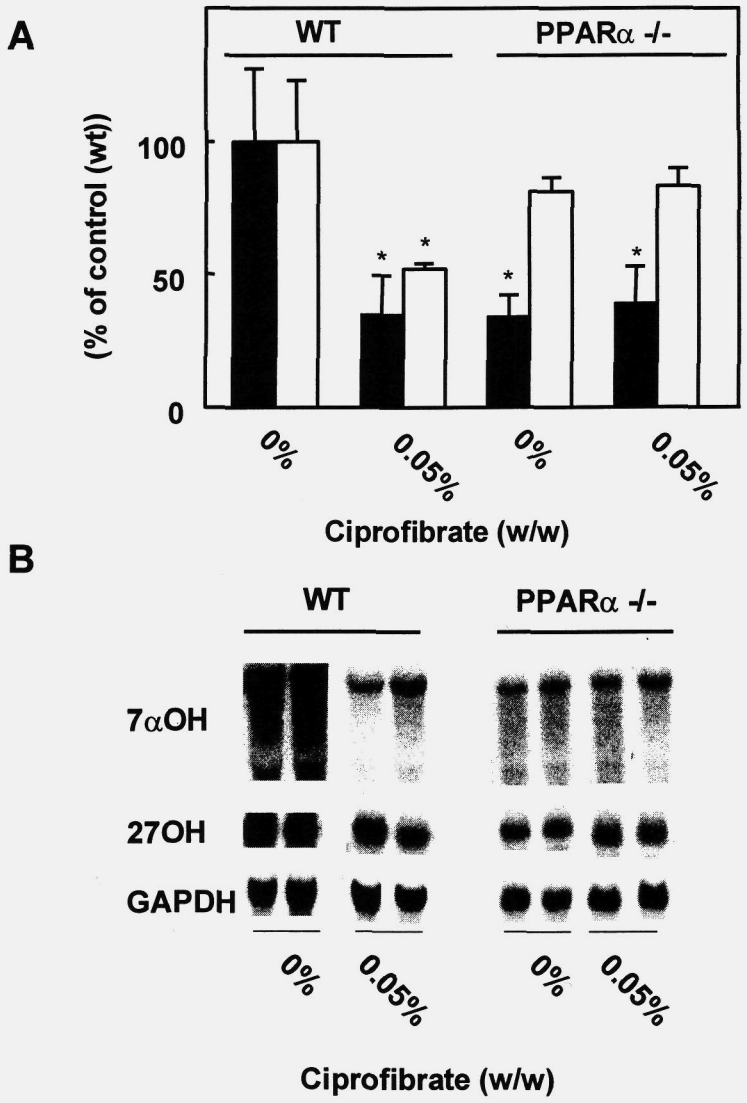

Fig. 4 Effect of ciprofibrate on cholesterol 7α-hydroxylase and sterol 27-hydroxylase mRNA in wild-type and PPARα -/- mice

(A) Cholesterol 7α-hydroxylase (closed bars) and sterol 27-hydroxylase (open bars) mRNA levels were measured in wild-type and PPARα -/- mice treated with or without ciprofibrate. Values shown are expressed as a percentage of values obtained in wild-type mice on control (chow) diet and are means ± S.D. of 4 mice. The amount of mRNA was corrected for differences in total RNA applied to the gel, using GAPDH as an internal standard. A significant difference is indicated by an asterisk (* $P<0.01$ compared with wild-type mice treated without ciprofibrate). (B) Representative Northern blot analysis of cholesterol 7α-hydroxylase (7αOH), sterol 27-hydroxylase (27OH) and GAPDH.

responsive to a variety of physiological signals. To assess whether this particular region of the cholesterol 7α-hydroxylase promoter is also responsive to PPARα, transfection experiments were performed, using the -376/+32 sequence of the rat cholesterol 7α-hydroxylase promoter fused to the luciferase-reporter gene (pGL3-376) (30). In cells co-transfected with PPARα, promoter activity of this construct was dose-dependently decreased in the presence of increasing concentrations of ciprofibrate or Wy14,643 as compared to control incubations (Fig 5).

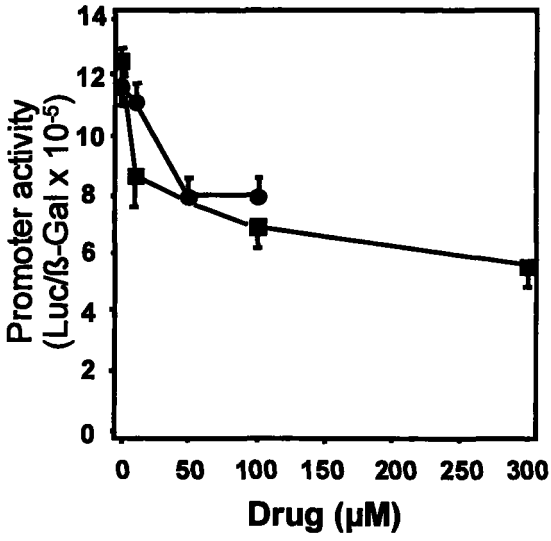

Fig. 5 Effect of PPARα activators on the transcriptional activity of the rat cholesterol 7α-hydroxylase promoter. HepG2 cells were cotransfected with the reporter plasmid pGL3-376Luc and the expression vector for PPARα. Transfection was carried out with 750ng of pGL3-376Luc as a promoter/reporter gene, 50ng of pCMVß, to adjust for transfection efficiency and 250ng of the expression vector for PPARα. Transfected cells were treated with increasing concentrations of Wy14,643 (circles) and ciprofibrate (squares) or 0.1% DMSO (control). Normalized luciferase activities are expressed as mean ± S.D. of triplicate samples.

To identify a PPARα-responsive site in the cholesterol 7α-hydroxylase gene, site-directed mutagenesis was performed (Fig 6). Because PPAR/RXR heterodimers bind to a DR-1 sequence, we mutated the 5' half-site of the DR-1 motif (pGL3-376m11) located at -146/-141. This DR-1 sequence is also reported as the response sequence for the nuclear receptor hepatocyte nuclear factor-4 (HNF-4) (30). HNF-4 is a transcription factor involved in the basal expression of cholesterol 7α-hydroxylase (30) and previous studies reported that PPARα may interfere with HNF-4-mediated activation of gene expression resulting in the suppression of gene transcription (39,40). This mutation reduced basal transcriptional activity of this chimeric gene to a much lower level than that of the wild-type promoter chimeric construct (pGL3-376). Mutation of the downstream located 3' half-site of the overlapping DR-5 motif (-128/-123) (pGL3-376m13) stimulated the reporter gene

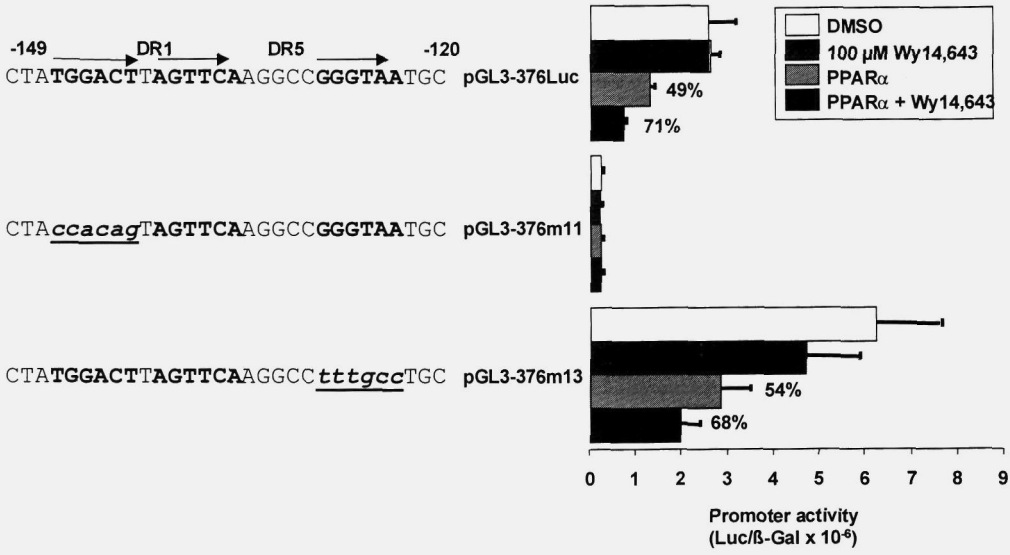

Fig. 6 Identification of the promoter sequence involved in PPARα-mediated down-regulation of cholesterol 7α-hydroxylase transcriptional activity

Confluent HepG2 cultures were grown as described in the experimental section. Transfection was carried out with 750ng of pGL3-376Luc or its mutants m11 or m13 as promoter/reporter genes, 50ng of pCMVß, to adjust for transfection efficiency with or without the expression vector for PPARα (250ng) and the PPARα agonist Wy14,643 (100 µM) as indicated. Total plasmid amount was kept constant by adding the empty expression vector. Normalized promoter activities are expressed as mean ± S.D. of triplicate samples. The percentages of inhibition of the cholesterol 7α-hydroxylase promoter activity due to PPARα overexpression and Wy14,643 treatment are shown where appropriate.

activity, as reported previously (30). Mutation of the DR-1 half-site also completely abolished the repressive effect of overexpressing PPARα and the addition of Wy14,643, representative for the existence of a PPARα responsive site. However, mutation of the 3' half-site of the DR-5 motif did not show this effect, indicating that the DR-5 is not involved in the PPARα-induced suppression (Fig 6). These results indicate that the DR-1 motif as well as the DR-5 motif are involved in the basal activity of the proximal promoter and that the DR-1 motif contains a negative PPRE.

Since the localized PPRE coincides with the binding site for HNF-4, we investigated the interaction of PPARα with HNF-4-mediated stimulation of cholesterol 7α-hydroxylase gene transcription. Cells were cotransfected with the wild-type reporter plasmid pGL3-376Luc and the expression vectors for HNF-4 and/or PPARα. HNF-4 stimulated the promoter activity 2.5-fold. However, this increase was abolished by co-transfection with PPARα and further decreased by incubation with

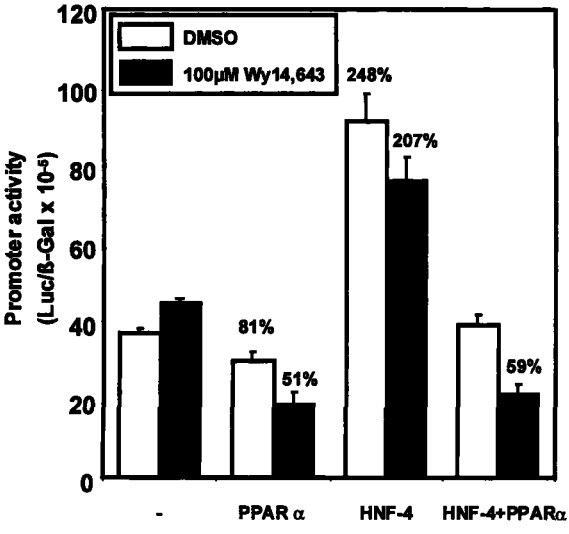

Fig. 7. Effect of PPARα on the HNF-4-mediated activation of cholesterol 7α-hydroxylase transcriptional activity.
Confluent HepG2 cells were cotransfected with 750ng of the reporter plasmid pGL3-376Luc and the indicated combinations of HNF-4 and PPARα expression vectors (250ng each). The total amount of plasmid was kept constant by adding the empty expression vector. Transfected cells were treated with 100µM Wy14,643 or an equivalent concentration of vehicle (0.1% DMSO). Normalized data are expressed as mean ± S.D. of triplicate samples. The numbers above the value bars indicate the percentages of promoter activity relative to the control sample cotransfected with empty expression vector and treated with vehicle alone.

Wy14,643 below control levels (Fig. 7). Thus, PPARα also interferes with HNF-4 mediated activation of cholesterol 7α-hydroxylase.

DISCUSSION

In this study, we showed that fibrates decrease bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression in rodents. Activation of the nuclear hormone receptor PPARα by fibrates mediated the suppression of gene expression of both genes. A direct involvement of PPARα was provided using PPARα-/- mice. Promoter-reporter studies indicated the presence of a functional PPARα-responsive element in the proximal promoter of the cholesterol 7α-hydroxylase gene and/or interference with HNF-4-mediated activation of cholesterol 7α-hydroxylase gene expression, both mediating suppression by fibrates.

Our finding that fibrates suppress cholesterol 7α-hydroxylase gene expression in rats and mice parallels with human studies showing a lower enzyme activity of cholesterol 7α-hydroxylase concomitantly with a decrease in the amount of bile acids excreted during fibrate treatment (11,22). Additionally, we found that down-regulation of sterol 27-hydroxylase may also contribute to the reduction in bile acid synthesis. Whether sterol 27-hydroxylase is also reduced in humans treated with fibrates is not

yet known. However, the observation that the relative amount of cholate in the bile of humans treated with fibrates is increased (11) suggests that the acidic pathway, initiated by sterol 27-hydroxylase, is also affected. Nevertheless, a decreased bile acid synthesis after fibrate treatment in humans can contribute to the observed increase in lithogenicity index (8-10).

The down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase, was not a specific effect of ciprofibrate, but rather a general characteristic of fibrates, since all the fibrates tested showed suppression of both genes. Among the compounds tested gemfibrozil appeared to be less active in the suppression of mRNA levels of both enzymes. In contrast, no effect was observed with the high-affinity ligand for PPARγ, the thiazolidinedione BRL49653, indicating that PPARγ is not involved in the repression of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression. It should be noted, however, that PPARγ levels are low in the liver (7).

Whereas the effects of fibrates obtained in cultured rat hepatocytes on cholesterol 7α-hydroxylase enzyme activity and mRNA levels were comparable *in vivo* in rat, we did not detect significant changes in sterol 27-hydroxylase by ciprofibrate in rat. The reason for this discrepancy is unknown, but the finding was consistently made in all rats and may be related to a species difference in the sensitivity of the sterol 27-hydroxylase gene towards the effects of fibrates *in vivo.* In contrast, wild-type mice responded well to ciprofibrate treatment by suppression of both cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression. This effect of ciprofibrate was completely abolished in PPARα-/- mice, showing the direct involvement of PPARα in the down-regulation of both genes by fibrates. No difference in the basal expression of the sterol 27-hydroxylase mRNA levels was observed by removing PPARα from the organism, suggesting that PPARα is not involved in modulating the basal sterol 27-hydroxylase expression. This indicates that endogenous ligands, known to be activators of PPARα, such as long-chain fatty acids (palmitic acid, linoleic acid and arachidonic acid) and eicosanoids (leukotriene B_4, 8(S)-hydroxyeicosatetraenoic acid), (33,41) are not active in PPARα-mediated down-regulation of sterol 27-hydroxylase gene expression. In contrast, knocking out PPARα resulted in a 3-fold diminution of basal cholesterol 7α-hydroxylase mRNA levels, suggesting that PPARα is involved in maintaining basal cholesterol 7α-hydroxylase expression. How this would happen is not yet known. An explanation may be that PPARα is involved in the sequestration of a (co)repressor, in analogy as

described in ref. 42, that negatively regulates cholesterol 7α-hydroxylase gene transcription and/or that PPARα may interfere with other nuclear transcription factors mediating their effect by binding to the same responsive site as PPARα, e.g. factors involved in bile acid-mediated suppression (30,37). We have shown that the PPRE fully coincides with the previously reported bile acid response element (BARE) II (37). Further studies are necessary to elucidate this phenomenon.

A direct involvement of PPARα in the expression of different genes in lipid metabolism has been reported showing suppression as well as induction of gene transcription. Whereas among others (reviewed in 1) the genes encoding human apolipoprotein AI and AII, and the genes involved in fat metabolism (lipolysis of triglycerides and uptake and ß-oxidation of fatty acids) are positively regulated, the human apolipoprotein CIII gene and genes involved in the synthesis of fatty acids are negatively regulated. Our finding that cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression is decreased upon activation of PPARα indicates that PPARα is not only directly involved in the metabolism of triglycerides and fatty acids but is also involved in the metabolism of cholesterol, although in the opposite way.

Several studies reported the presence of different closely situated elements in the proximal cholesterol 7α-hydroxylase promoter, which are responsive to various physiological signals (30,31,34-38). Our transfection experiments have now also identified an active PPARα-responsive element in this region. Site-directed mutagenesis revealed that the DR-1 (-146/-134), is involved in maintaining the basal activity of the promoter and that this element is negatively regulated by activated PPARα. In contrast, the overlapping downstream-localized DR-5 sequence (-139/-123), showed as expected, no involvement in the PPARα-mediated regulation by fibrates, but contributed to the basal transcriptional activity of the cholesterol 7α-hydroxylase gene, although in an opposite way to the DR-1 sequence.

Interestingly, the now identified PPRE coincides with the recently mapped bile acid response element (BARE) II (37), which is also reported as the response sequence for HNF-4 (30). HNF-4 is a transcription factor involved in the basal expression of cholesterol 7α-hydroxylase (30). We showed that PPARα also interferes with the HNF-4 mediated activation of cholesterol 7α-hydroxylase gene transcription. Thus, in addition to direct negative regulation via the PPRE, the PPARα-mediated transcription of cholesterol 7α-hydroxylase may also be suppressed by means of competition with HNF-4 for the DNA binding site (39). Otherwise, negative regulation of the expression of cholesterol 7α-hydroxylase could

be exerted by down-regulation of HNF-4 expression, as has been shown for fibrate action in other negatively-regulated genes (39,40). Further experiments, including functional analysis of the regulatory regions of the gene encoding cholesterol 7α-hydroxylase will be necessary to elucidate the precise mechanism of transcriptional repression of cholesterol 7α-hydroxylase gene expression by PPARα.

So far, the sterol 27-hydroxylase promoter has not been functionally characterized. The co-ordinate transcriptional regulation of both enzymes by the same mediators as shown in this and other studies (18,26,38,43,44) is indicative of similar binding of nuclear factors to several putative binding sequences in the promoter region of both these enzymes. Further studies should elucidate whether the sterol 27-hydroxylase promoter also harbors a functional PPARα responsive site.

In conclusion, we found that fibrates inhibit bile acid synthesis by down-regulating cholesterol 7α-hydroxylase and sterol 27-hydroxylase, which is mediated via activation of PPARα. Suppression of bile acid synthesis may contribute to the formation of gallstones in patients after long-term therapy with fibrates.

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Chapter 7

Differential effects of 17α-ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat.

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Differential effects of 17α-ethinylestradiol on the neutral and acidic pathways of bile salt synthesis in the rat

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Running title: effects of 17α-ethinylestradiol on bile salt synthesis.

ABSTRACT

Effects of 17α-ethinylestradiol (EE) on the neutral and acidic biosynthetic pathways of bile salt (BS) synthesis were evaluated in rats with an intact enterohepatic circulation and in rats with long-term bile diversion to induce BS synthesis. For this purpose, bile salt pool composition, synthesis of individual BS *in vivo*, hepatic activities and expression levels of cholesterol 7α-hydroxylase (CYP7A) and sterol 27-hydroxylase (CYP27) as well as of other enzymes involved in BS synthesis were analyzed in rats treated with EE (5 mg/kg, 3 days) or its vehicle. BS pool size was decreased by 27% but total BS synthesis was not affected by EE in intact rats. Synthesis of cholate was reduced by 68% in EE-treated rats, while that of chenodeoxycholate was increased by 60%. The recently identified Δ^{22}-isomer of β-muricholate contributed for 5.4% and 18.3 % ($p<0.01$) to the pool in control and EE-treated rats, respectively, but could not be detected in bile after exhaustion of the pool. A clear reduction of BS synthesis was found in bile-diverted rats treated with EE, yet, biliary BS composition was only minimally affected. Activity of CYP7A was decreased by EE in both intact and bile-diverted rats, whereas the activity of the CYP27 was not affected. Hepatic mRNA levels of CYP7A were significantly reduced by EE in bile-diverted rats only; CYP27 mRNA levels were not affected by EE. In addition, mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase were increased by bile diversion and suppressed by EE. This study shows that EE-induced intrahepatic cholestasis in rats is associated with selective inhibition of the

neutral pathway of BS synthesis. Simultaneous impairment of other enzymes in the BS biosynthetic pathways may contribute to overall effects of EE on BS synthesis.

INTRODUCTION

The synthetic estrogen 17α-ethinylestradiol (EE) induces cholestasis in rodents, mainly by reducing the bile salt-independent fraction of bile flow (BSIF) (1,2). The mechanisms underlying the decrease in bile flow are not yet clear (3). In addition to reduced BSIF, several authors have reported that EE may also affect bile salt-dependent bile formation (BSDF) (2,4,5). This effect has been attributed to reduced hepatic BS synthesis (4-6) and to impaired activities of hepatic transport systems involved in vectorial transport from blood to bile (7-9). With respect to the first, reduced biliary BS secretion and altered biliary BS composition in EE-treated animals have been reported (4,5,10). In particular, the contribution of chenodeoxycholate and β-muricholate to the BS pool appears to increase at the expense of cholate. In a previous study (10), we found that conversion of endocytozed lipoprotein cholesterol to cholate was completely abolished in EE-treated rats. The metabolic basis for the changes in BS synthesis induced by EE are largely unknown. Earlier studies have shown that EE inhibits the activity of the cholesterol 7α-hydroxylase (CYP7A) (11,12), the enzyme catalyzing the first step of the so-called neutral pathway of BS biosynthesis. Since then, however, it has become clear that an acidic pathway, initiated by 27-hydroxylation of cholesterol by the mitochondrial sterol 27-hydroxylase (CYP27), represents a quantitative important route for BS synthesis (13-15). Recently, data have been reported to indicate that in situations where the "classical" neutral pathway is specifically suppressed, the acidic pathway becomes more important for maintenance of hepatic BS synthesis (16,17). It is not known whether EE differentially affects both pathways. In addition, peroxisomal formation of a Δ^{22} isomer of muricholate has been suggested as a novel further downstream pathway in BS synthesis in rats (18,19). Whether and to what extent formation of this species is affected under cholestatic conditions is not known. Finally, EE has strong impact on hepatic cholesterol synthesis (e.g., 20-23). As the contribution of newly synthesized cholesterol to formation of individual BS may vary under different conditions (24-26), altered cholesterol synthesis may also affect BS synthesis.

To assess the quantitative contribution of the major pathways to hepatic BS synthesis in EE-treated rats we related *in vivo* BS synthesis to the specific activities and expression levels of CYP7A and CYP27. In addition, mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase, key enzymes in the formation of cholate and β-muricholate, respectively, were determined, as well as those of HMG-CoA synthase as a key enzyme in cholesterol synthesis. Experiments were performed in rats with an intact enterohepatic circulation and in rats with prolonged bile diversion. Bile diversion leads to pool depletion and to upregulation of hepatic BS synthesis, thereby enabling us to directly assess the effects of EE on synthesis of the individual BS species and to relate these effects to hepatic enzyme activities.

MATERIALS AND METHODS

Materials

17α-Ethinylestradiol (EE) was purchased from Sigma Chemicals (St. Louis, MO, USA). NADPH, isocitrate-dehydrogenase was obtained from Boehringer Mannheim (Mannheim, Germany). Cholesterol oxidase was obtained fron Calbiochem (La Jolla,CA, USA). All other chemicals were of reagent grade or the highest purity commercially available.

Animals

Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands) weighing 290-330 g were used for these studies. Animals were kept in a light- and temperature-controlled environment and had free access to lab chow and tapwater throughout the experiments. The animals received humane care and experimental protocols complied with the local guidelines for use of experimental animals.

To study the effects of EE on bile formation and composition under conditions with an intact enterohepatic circulation, rats were equipped with permanent catheters in bile duct and duodenum as described in detail elsewhere (27). Both catheters were immediately connected to each other to maintain an intact enterohepatic circulation. Subcutaneous EE (5 mg/kg) or solvent (1,2-propanediol) injections were given for 3 days, starting four days after surgery, i.e., after animals had regained their preoperative body weights. After three days of treatment, the connection between both catheters was interrupted and bile was collected for 6 hours in 30 min intervals by means of a fraction collector. Bile volume was determined gravimetrically and samples were immediately stored at -20 °C for later analysis. Separate groups of rats were used for the isolation of hepatic microsomes, mitochondria, total RNA and for collection of blood.

In order to study the effects of EE on bile formation and composition after long-term bile diversion, when BS synthesis is maximally upregulated, rats where equipped with a permanent catheter in the bile duct only. Bile was diverted for 5 days, prior to administration of EE or the solvent for three days. These animals were allowed to drink 0.9% NaCl to compensate for loss of electrolytes via bile. At day 8, bile samples were continuously

collected by means of a fraction collector for 24 hr in 90 min intervals. After the bile sampling, the animals were anesthetized with halothane. Blood was sampled by means of a cardiac puncture and the liver was removed for isolation of total RNA, microsomes and mitochondria.

Analyses

BS in plasma and bile were determined by an enzymatic fluorimetric assay (28). Plasma triglycerides, plasma- and hepatic cholesterol were measured enzymatically using commercially available kits (Boehringer Mannheim, Mannheim, Germany). Aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin in plasma were assessed by standard laboratory techniques.

Bile salt composition was studied by gas chromatography and gas chromatography / mass spectrometric techniques as described earlier for human bile (29). Briefly 5 - 50 µl bile was subjected to enzymatic hydrolysis with cholylglycine hydrolase. The free bile acids formed were extracted with C18 solid phase extraction, methylated and silylated. The methyl-TMS derivatives were separated on a 25m x 0.25 mm OV-1701 column (CP Sil19 CB, Chrompack Int., Middelburg, The Netherlands). As a modification coprostanol was used as internal standard for the purpose of quantitation applying GC only. Identification of bile acids was performed by GC/MS (SSQ7000, Finnigan MAT, San Jose, CA, U.S.A.) using the same GC separation system. Full scan data were recorded from m/z 50 - 850 and mass spectra were compared with reference spectra for definitive identification. In the absence of reference spectra, a tentative identification was done based on spectral information (19).

Preparation of microsomes and mitochondria

For the isolation of microsomes and mitochondria, livers were perfused with cold saline, removed and 5 gram of liver tissue was stored in 250 mM sucrose, 10 mM Tris, 1 mM EDTA, pH 7.4. All procedures were carried out at 4°C. Livers were cut into small pieces with scissors and homogenized in the same buffer using a potter. The homogenate was centrifuged for 10 min at 800 g, and the supernatant was then centrifuged 12 min at 8500 g. The thus obtained supernatant was used for isolation of microsomes and the pellet for the isolation of mitochondria.

For the isolation of microsomes the supernatant was centrifuged for 70 min at 100000 g. The pellet was resuspended by means of a potter in 100 mM sucrose, 100 mM potassium phosphate, 2 mM EDTA and 5 mM DTT, pH 7.4 and centrifuged for 1 h at 100000 g. Microsomes were resuspended in the same buffer and frozen quickly in fluid N_2 in small aliquots and stored at -80°C.

The mitochondrion-enriched pellet was resuspended by means of a potter in 250 mM sucrose, 10 mM Tris , pH 7.4 and centrifuged for 12 min at 8500 g. This procedure was repeated 3 times. The final mitochondrial pellet was resuspended in this buffer and stored at -80°C. Protein concentration was measured according to Lowry (30)

Assay of cholesterol 7α-hydroxylase and sterol 27-hydroxylase enzyme activity

Enzyme activities of CYP7A and CYP27 in isolated liver microsomes and mitochondria were determined essentially according to Chiang (31) measuring conversion of cholesterol into 7α- and 27-hydroxycholesterol, respectively. In short, 750 µg of protein of either microsomal or mitochondrial protein was incubated in 1 ml of buffer containing 0.1 M potassium phosphate

pH 7.2, 50 mM NaF, 5 mM DTT, 1 mM EDTA, 20% glycerol (w/v) and 0.015 % (w/w) CHAPS. Twenty µl of 1 mg cholesterol in 45% (w/v) hydroxypropyl-β-cyclodextrin was added and the mixture was incubated with agitation for 10 min at 37 °C. Then 200 µl of a regenerating system was added containing 10 mM sodium isocitrate, 10 mM $MgCl_2$, 1 mM NADPH and 0.15 U isocitrate-dehydrogenase at 37 °C. After 20 min of incubation 60 µl of a stop solution containing 20% sodium cholate and 1µg 20α-hydroxycholesterol, which served as an internal standard, was added. Steroid products were oxidized for 45 min with 100 µl buffer containing: 0.1 % cholesterol oxidase, 10mM K_2HPO_4, 1 mM DTT and 20% glycerin at 37 °C and the reaction was stopped by addition of 2 mL ethanol. Cholesterol metabolites from this reaction mixture were extracted in petroleumether and the ether layer was evaporated under a stream of nitrogen. Residues resuspended in a mixture of 60% acetonitril, 30% methanol and 10% chloroform (v/v) were analyzed by using C-18 reverse phase HPLC on a Tosohaas TSK gel-ODS 80TM column equilibrated with 70% acetonitril and 30% methanol at a flow rate of 0.8 ml/min. The amount of products formed was determined by monitoring theabsorbance at 240 nm. Peaks were integrated using Data Control software (Cecil Instruments, UK).

Determination of mRNA levels

Total RNA was isolated according to Chomczynski (32). Determination of steady state mRNA levels for CYP7A, CYP27, 12α-hydroxylase, lithocholic acid 6β-hydroxylase, HMG-CoA synthase and LDL receptor by Northern blot and dot blot and hybridization conditions were performed as described previously (33-35). 18S ribosomal RNA was used as an internal standard to correct for differences in amounts of total RNA applied to the gel. mRNA levels were quantified by phospho-imager analysis (Fuij Fujix Bas 1000) by using the program TINA version 2.08c.

Calculations and statistics

Output rates of BS were determined by multiplying bile flow with BS concentrations, after correction for the dead space of the tubing system. Values are expressed as mean ± SD. Significance of difference between two groups was assessed by means of Mann-Whitney nonparametric test at $p<0.05$ level of significance.

RESULTS

Animal characteristics

There were no significant differences in body weight between EE- or solvent-injected intact or bile-diverted rats (Table 1). The liver-to-body weight ratio increased significantly upon EE treatment in both conditions. Table 2 shows the effects of EE treatment for three days on plasma markers of liver function. Aspartate transaminase, alanine transaminase and bilirubin levels in plasma were not significantly affected by EE. The plasma BS concentration was significantly increased in intact rats upon EE treatment. As expected, BS levels were at the lower limits of detection in the untreated bile-diverted rats and did not increase after EE

administration. Treatment with EE led to significant reductions in plasma cholesterol and triglyceride levels in both experimental models.

Table 1 Body- and liver weights in control and EE-treated intact and bile diverted rats.

	Body weight (g)	Liver weight (g)	Liver (% Body weight)
Intact, control	325.0 ± 49.4	14.2 ± 2.6	4.3 ± 0.2
Intact, EE	332.6 ± 39.0	17.5 ± 2.5	5.3 ± 0.2*
Bile diverted, control	322.0 ± 23.9	13.4 ± 0.9	4.2± 0.3
Bile diverted, EE	298.3 ± 24.3	14.3 ± 0.7	4.8 ±.0.4*

Intact and bile diverted rats were treated with EE (5mg/kg) for three consecutive days. At 24 h after the last injection, animals were weighed, anesthesized with halothane and a large blood sample was collected by cardiac puncture. Subsequently the liver was removed and weighed.Data are given as means ± SD, n=3-5 per group
* significantly different ($p < 0.05$) from respective control

Table 2. Effect of EE treatment for 3 days on plasma markers in intact and bile diverted rats.

	AST (IU/L)	ALT (IU/L)	Bilirubin (µM)	Bile Salt (µM)	Cholesterol (mM)	Triglyceride (mM)
Intact, control	89 ± 16	41 ± 2	5.5 ± 1.7	36 ± 12	1.33 ± 0.66	1.34 ± 0.66
Intact, EE	115 ± 50	40 ± 11	5.2 ± 1.1	77 ± 22 *	0.32 ± 0.05 *	0.21 ± 0.11 *
Bile diverted, control	222 ± 81	52 ± 14	8.3 ± 5.9	4 ± 0	1.39 ± 0.35	1.42 ± 0.65
Bile diverted, EE	112 ± 74	30 ± 6	4.8 ± 0.5	2 ± 1	0.39 ± 0.14 *	0.33 ± 0.27 *

See legend table 1 for experimental details. Data are given as means ± SD, n=3-5 per group
* significantly different $p < 0.05$ from respective control

Bile formation

Figure 1A shows bile flow during 6 hours following interruption of the enterohepatic circulation of intact rats (left panel) and during a 24 hr period after 8 days of bile diversion (right panel). EE treatment significantly decreased bile flow in intact rats, which, as shown previously (3), is mainly caused by reduction of the BSIF. EE also

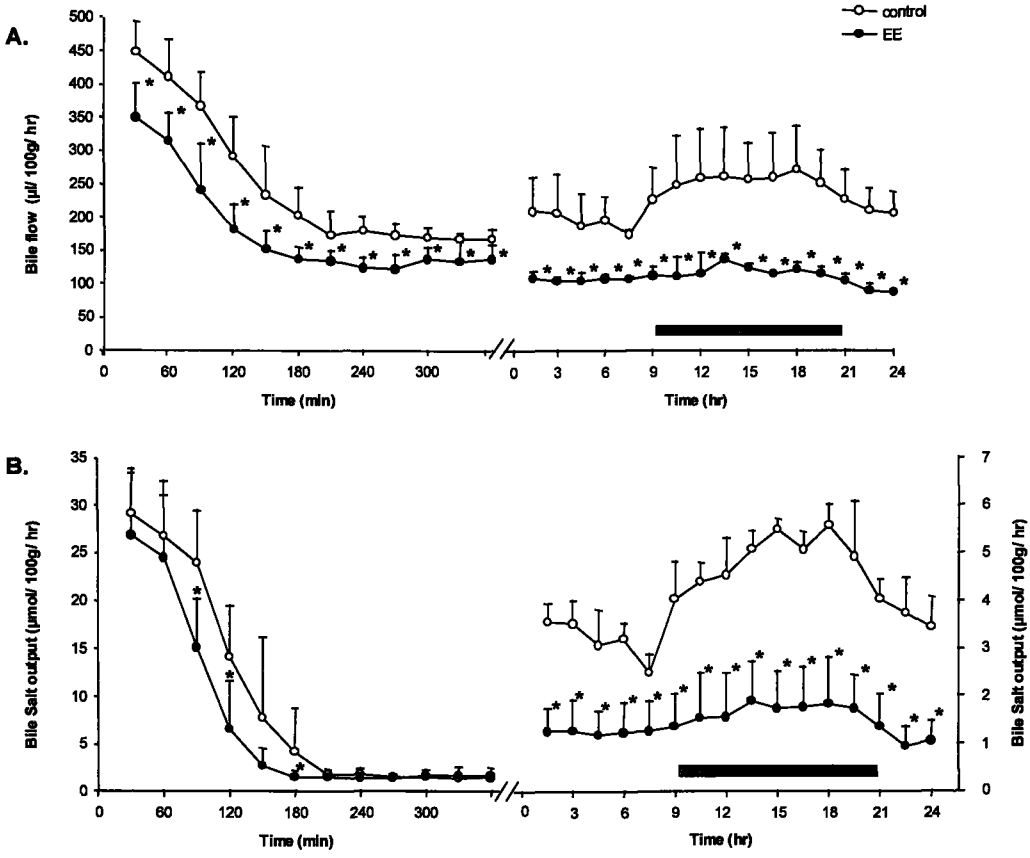

Fig 1. The effect of EE on bile flow (A) and BS output (B) in intact rats during 6 h following interruption of the enterohepatic circulation (left panel) and the effect of EE during 24 h after 8 days of bile diversion (right panel). In intact rats bile was collected in 30 min fractions for 6 hours after interruption of the enterohepatic circulation. In 8 day bile-diverted rats bile was collected continuously during 24 hours in 90 min fractions. Horizontal bars indicate the dark period. Values are means ± SD of 3-6 rats per group.
* significantly different from respective control ($p<0.05$)

decreased bile flow in rats with long-term bile diversion; In this condition, the well-established diurnal variation in bile flow was absent. BS output was decreased upon EE treatment in both intact and bile-diverted rats (Figure 1B). BS poolsizes, as calculated from the wash-out curves in the intact rats were 53.0 ± 17.2 µmol/100g in the controls and 37.0 ± 12.9 µmol/100g in the EE-treated rats respectively ($p < 0.05$). The BS synthesis rate, determined at the nadir of the wash-out curve, was not affected by EE treatment, i.e. 1.54 ± 0.16 µmol/h/100g and 1.57 ± 0.16 µmol/h/100g in control and EE-treated rats, respectively. After 8 days of bile diversion, BS synthesis rate increased to 3.37 ± 0.52 µmol/h/100g in control rats at day time, whereas in the EE-treated bile-diverted animals synthesis was significantly lower, i.e., 1.23 ± 0.04 µmol/h/100g. This figure also shows that the characteristic increase in BS output during the dark period in bile-diverted control rats (36) was absent in the EE-treated rats. These results imply that, after long-term bile diversion, the estrogen also impairs the magnitude of the BSDF.

Table 3. The effect of EE-treatment on bile salt pool composition and on composition of newly synthesized bile salts immediately after exhaustion of the circulating pool.

BS species	BS Pool		BS Synthesis	
	control (%)	EE (%)	control (%)	EE (%)
LC	1.1 ± 0.2	1.5 ± 0.6	2.8 ± 1.1	1.8 ± 1.3
DC	3.5 ± 1.3	5.2 ± 3.2	11.0 ± 3.1	15.5 ± 7.4
C	67.0 ± 3.7	50.8 ± 12.2 *	36.9 ± 4.6	11.8 ± 1.7 **
CDC	9.6 ± 3.5	8.3 ± 4.4	20.9 ± 4.5	33.4 ± 8.3 **
HDC	5.9 ± 1.9	8.8 ± 3.8	14.0 ± 3.7	18.1 ± 2.8
UDC	2.2 ± 1.0	0.2 ± 0.5 **	0.9 ± 0.4	1.6 ± 0.9
β-MC	5.5 ± 2.8	7.0 ± 1.9	12.1 ± 2.4	16.1 ± 4.8
Δβ22MC	5.4 ± 1.7	18.3 ± 3.8 **	1.2 ± 0.5	1.7 ± 0.7

After interruption of the enterohepatic circulation, 30 min bile samples were collected. Pool composition was determined in the 0-30 sample. Synthesis was measured at 300- 330 min after the interruption i.e., after exhaustion of the BS pool. Data are given as means ± SD, n= 4-5 per group

* *significantly different* ($p < 0.05$),

** ($p<0.01$) from respective control

The relative contribution of the individual BS present in the pool was assessed by GC and GC-MS analysis (Table 3). In the BS pool, the contribution of cholate was decreased from 67.0 ± 3.7 to 50.8 ± 12.2 %, probably caused by a decreased cholate synthesis, as determined after pool depletion. On the other hand, synthesis of CDC was relatively increased by EE. Secondary BS, i.e., DC and LC were present in bile in low concentrations after 6 h pool depletion, probably reflecting their slow entry into the bloodstream from the colon. Their contribution was not affected by EE. The recently identified (18,19) Δ^{22} isomer of β-muricholate comprised 5.4 % of the BS pool in control rats and 18.3 % in EE-treated rats. The identity of this unique rodent BS was confirmed by GC-MS, based upon the unique combination of fragment ions m/z 195, 285 (typical for muricholic acids) and 367, 456 and 546 (Figure 2) indicating a combination of a 3α,6α or 6β,7β- trihydroxy bile acid and a single double bond. According to Setchell *et al.* (19) the double bond is located at the C22 position. A similar Δ^{22} ω-muricholic acid could be identified based on the similar fragmentation pattern and the retention time shift relative to ω-muricholic acid which is comparable with the retention time shift of Δ22 β-muricholic acid relative to β-muricholic acid. After exhaustion of the pool, only traces of Δ^{22}-β-muricholate could be detected in bile of both control and EE-treated rats. After long-term bile diversion, the isomer was not detectable at all.

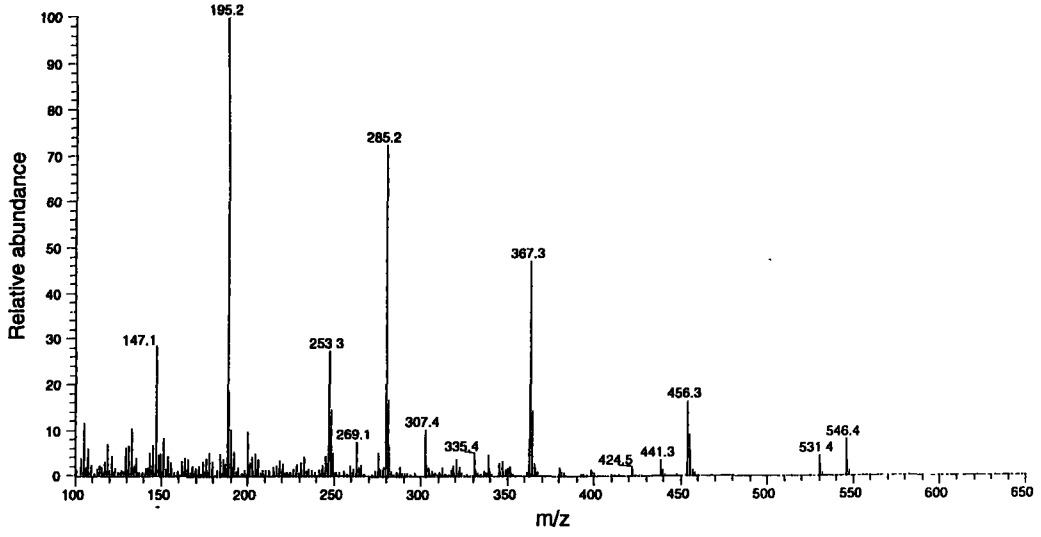

Fig 2. Mass spectrum of a bile acid present in rat bile tentatively identified as Δ 22-β-muricholic acid.

Table 4. The effect of EE on BS composition in long term bile diverted rats

BS species	control (%)	EE (%)
C	51.0 ± 7.2	46.3 ± 6.2
CDC	33.9 ± 3.2	28.4 ± 8.7
UDC	2.8 ± 0.9	4.0 ± 0.2
βMC	13.0 ± 5.9	21.2 ± 4.8

90 min bile samples were taken from long-term bile-diverted rats after 3 days of EE treatment. A sample at the mid-light period i.e. from 12-13.30 AM was used for determining BS composition. Data are given as means ± SD, n=3-5 per group

Table 4 shows BS composition in 8 day bile-diverted rats when BS synthesis is maximally upregulated (27). As the intestinal BS pool of these rats has been depleted, only primary BS are present in bile. The relative decrease in cholate synthesis seen in EE-treated intact rats was not found after long-term bile diversion. Also the other BS were not significantly affected by EE, although the relative contribution of β-MC tended to be increased by treatment with EE.

Activities and mRNA levels of cholesterol 7α-hydroxylase, sterol 27-hydroxylase, sterol 12α-hydroxylase and lithocholate 6β-hydroxylase

In order to gain insight into the metabolic background of the changes in BS composition induced by EE, the activities of the cholesterol 7α-hydroxylase and the sterol-27-hydroxylase were determined in isolated hepatic microsomes and mitochondria, respectively. Figures 3a and b show the activities of these enzymes, expressed as percentage of the solvent-treated intact controls. After 8 days of bile diversion, i.e., without administration of EE, the activity of CYP7A was 8-fold increased, as shown previously (37). Upon EE treatment, enzyme activity decreased from 2.87 ± 1.51 to 1.60 ± 0.34 nmol/mg/h ($p=0.065$) in intact rats (-44%) and from 22.94 ± 4.32 to 7.07 ± 3.18 nmol/mg/h ($p<0.05$) in bile diverted animals (-70%). It should be noted that the activity of CYP7A in the EE-treated bile-diverted rats was still increased compared with the control situation. After 8 days of bile diversion, the activity of CYP27 was increased by about 100%: EE treatment did not change activity of this enzyme.

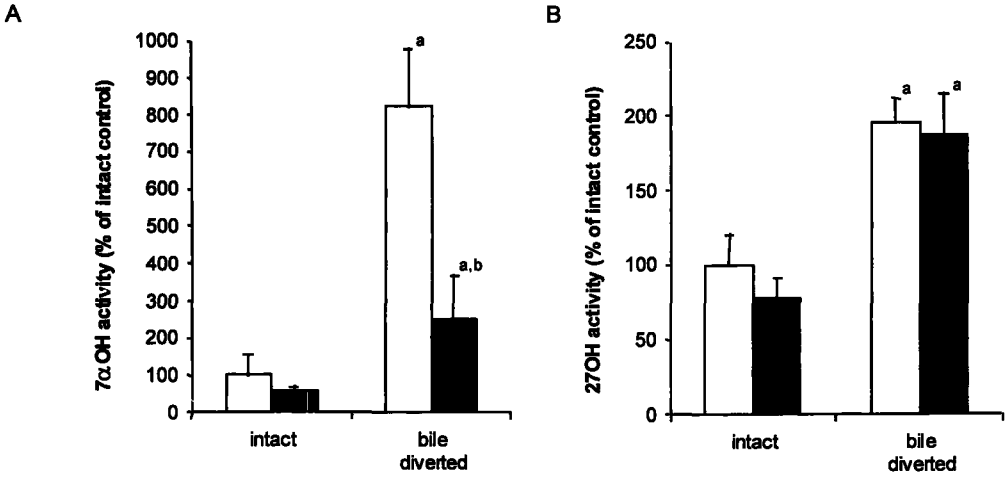

Fig 3. Microsomal cholesterol 7α-hydroxylase (a) and mitochondrial sterol 27-hydroxylase (b) activities in intact and bile-diverted solvent-treated (control) and EE-treated rats .
Microsomes and mitochondria were prepared from livers of the experimental groups harvested at 9 AM and enzyme activities were measured as described in the Materials and Methods section. White bars indicate solvent-treated control groups and black bars indicate EE-treated groups. Values are means ± SD for 3-6 rats per group and expressed as percentage of the untreated, intact control group. The 100% value is 2.78 ± 1.51 nmol/mg/hr for cholesterol 7α-hydroxylase and 1.28 ± 0.26 nmol/mg/hr for sterol 27-hydroxylase.
a = significantly different $p<0.05$ from intact control
b= significantly different $p<0.05$ from bile diverted control

To investigate the level of EE-interaction with BS synthesis, we determined steady state mRNA levels of *CYP7A* and *CYP27*. Figure 4 shows that mRNA levels of *CYP7A* and *CYP27* as well as of the LDL receptor, measured as an internal control signal, increased upon bile diversion. The *CYP7A* mRNA levels were not significantly lowered by EE in intact rats but clearly decreased in bile-diverted rats after treatment with the estrogen. *CYP27* mRNA levels, however, were not significantly affected by EE in either situation. The levels of LDL receptor mRNA were increased upon EE treatment both in intact and bile-diverted animals, as reported previously for intact rats (21,22,38,39).

The mRNA levels of sterol 12α-hydroxylase, essential for cholate synthesis, and of lithocholate 6β-hydroxylase, involved in β-muricholate formation, were clearly increased in bile diverted rats compared to intact animals and markedly down-regulated by EE-treatment (Figure 5). Furthermore, HMG-CoA synthase mRNA levels were increased after bile diversion, as expected, and clearly reduced by EE

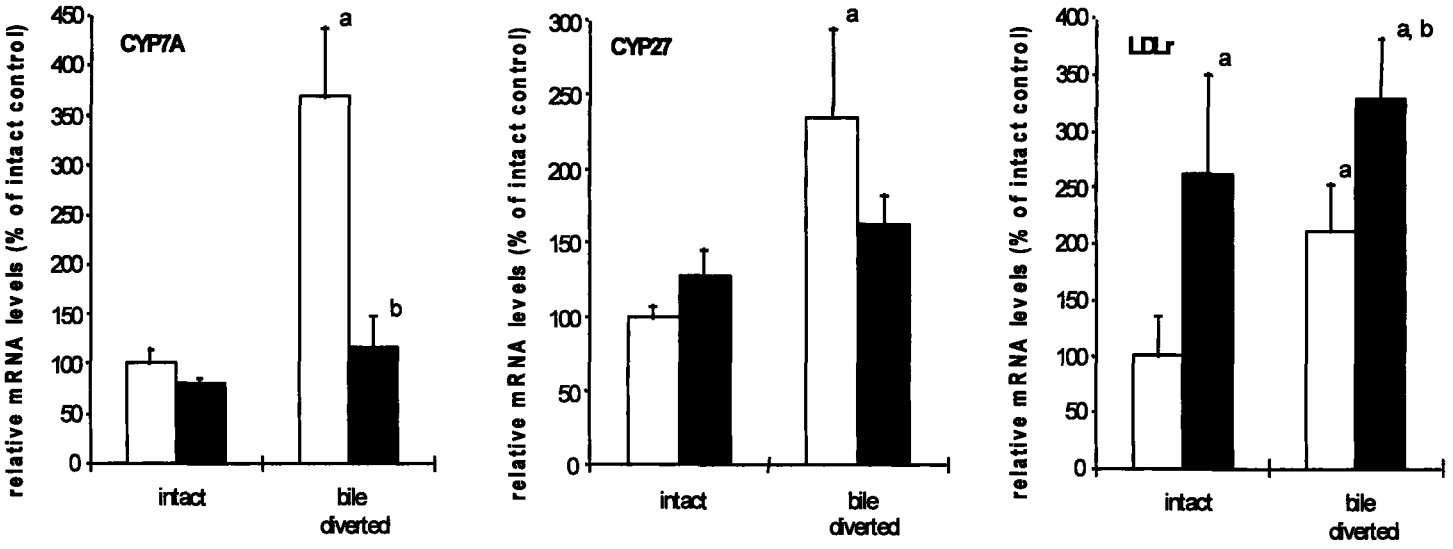

Fig 4. Relative mRNA levels of cholesterol 7α-hydroxylase (CYP7), sterol 27-hydroxylase (CYP27) and LDL receptor (LDLr) in intact and bile-diverted solvent-treated (control) and EE-treated rats

Liver material used for RNA isolation was harvested at 9 AM. White bars indicate solvent treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3-5 rats per group, expressed as percentage of the intact solvent-treated control group

a = significantly different $p<0.05$ from intact control.

b= significantly different $p<0.05$ from bile diverted control.

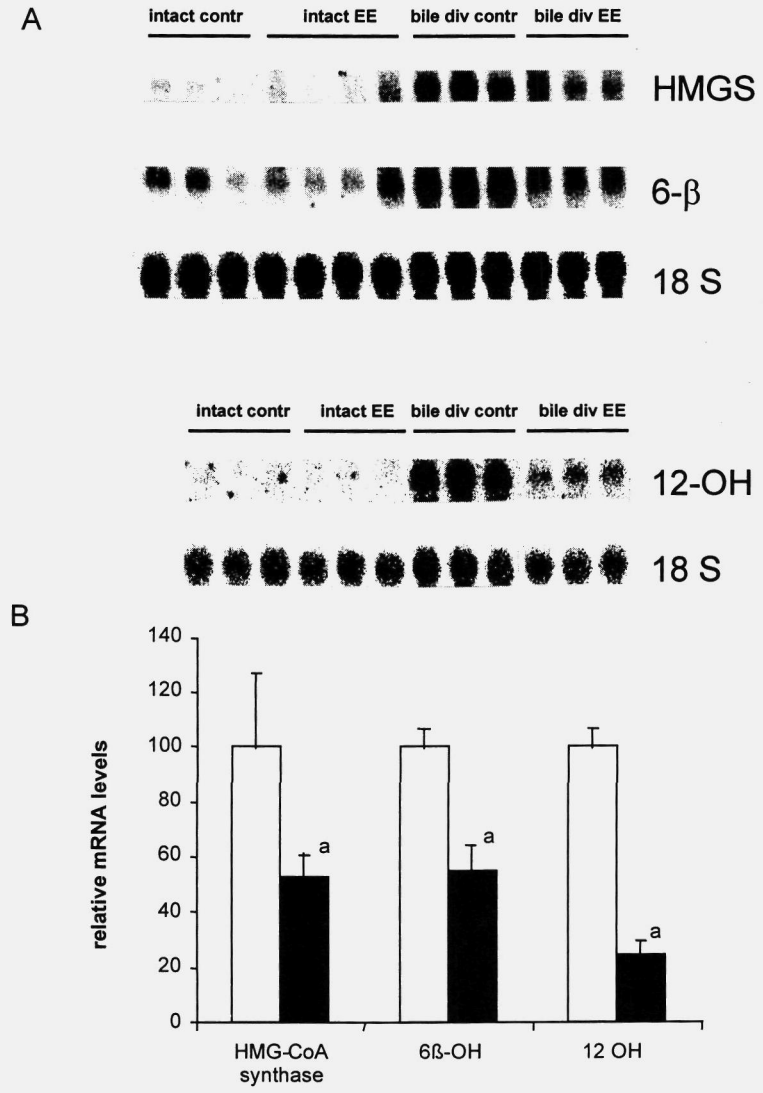

Fig 5. Northern blot analysis of lithocholate 6β-hydroxylase, sterol 12α-hydroxylase and HMG-CoA synthase in intact and bile diverted rats treated with solvent or EE (a) and relative mRNA levels in 8 day bile-diverted rats with and without EE treatment (b).

Liver material used for RNA isolation was harvested at 9 AM. White bars indicate solvent treated control groups and black bars indicate EE-treated groups. mRNA levels were quantified relative to 18S RNA signal. Data are mean ± SD for 3-5 rats per group, expressed as percentage of the intact solvent-treated control group

a = significantly different $p<0.05$ from intact control

treatment, confirming earlier reports (20,22) showing reduced cholesterol synthesis in EE-treated rats.

DISCUSSION

The effects of EE on BS synthesis and on expression and activity of key enzymes of the neutral and acidic pathways of BS synthesis were evaluated in rats under physiological conditions and in the situation with maximally upregulated BS synthesis (27). Rats were treated with the estrogen for 3 days. Based on previous experiments (3), we anticipated that, with this treatment schedule, changes observed would be attributable to EE rather than to secondary effects of full-blown cholestasis. The presence of cholestasis *per se*, for instance in the bile duct ligated rat (39, 40), strongly affects BS synthesis and CYP7A activity. Based on the minimal changes in plasma bilirubin and transaminases it can be concluded that, although bile flow was markedly reduced in EE-treated animals, there was no accumulation of bile components in the plasma nor was liver damage induced in this experimental set-up. Slightly elevated plasma BS concentrations in EE-treated intact rats probably reflect their increased spill-over to the systemic circulation due to down-regulation of the Na+-taurocholate cotransporting protein (Ntcp) (3, 8).

Results of the present study confirm earlier reports on the effects of long-term bile diversion on BS synthesis, CYP7A activity and *CYP7A* mRNA levels in rats (see 14, 15) and demonstrate that interruption of the enterohepatic circulation for 8 days also leads to a 2-fold increase in CYP27 activity and a 2.5 fold increase in *CYP27* mRNA levels. The latter results support previous findings in cholestyramine-fed rats and in cultured rat hepatocytes, demonstrating feed-back regulation of CYP27 by BS at a transcriptional level (25,42,43). In contrast to the situation in rats, CYP7A and CYP27 do not appear to be coordinately regulated by recirculating BS in the rabbit liver (17,44), delineating the remarkable inter-species differences in regulation of BS metabolism. In addition to the anticipated effects on *CYP7A* and *CYP27* as well as on *HMGS* expression it is shown for the first time that mRNA levels of sterol 12α-hydroxylase and lithocholate 6β-hydroxylase are markedly increased in the bile diverted rat, indicating that BS exert regulatory actions at multiple sites of their biosynthetic pathways. Alternatively, it may be that hepatic accumulation of BS precursors, due to increased activities of the rate-limiting enzymes, increases gene

transcription and/ or mRNA stability of enzymes catalyzing conversions further downstream in the biosynthetic cascade. It is also interesting to note that the well-established diurnal variation of BS synthesis in bile depleted rats (27) is completely abrogated by EE. As this diurnal rhythm is thought to be mediated by glucocorticoids (45) it is tempting to speculate that EE renders the BS synthetic cascade insensitive to stimulatory actions of glucocorticoids.

Both in intact rats and in bile-diverted animals, EE reduced CYP7A activity whereas *CYP7A* mRNA levels were reduced in the bile-diverted animals only. The fact that the decrease in enzyme activity just failed to reach statistical significance in intact rats in our hands is probably due to the relatively short treatment period: 5 days of treatment has repeatedly been shown to inhibit CYP7A activity in rat liver (11, 12). The divergent findings for *CYP7A* mRNA levels in intact and bile-diverted rats may point towards a differential effect of EE in both situations. In fact, Davis *et al.* (11) have provided evidence that EE may act directly on microsomal membranes and thereby inhibit CYP7A activity in livers of intact rats. On the other hand, in the derepressed state of BS synthesis in bile-diverted animals, the estrogen also acts at a pretranslational level. In marked contrast, EE had no effect on CYP27 activity or *CYP27* mRNA levels in either situation but mRNA levels of HMG-CoA synthase, sterol 12α-hydroxylase and lithocholate 6β-hydroxylase were also clearly suppressed by EE in the bile-diverted rats. Taken together, this suggests that EE affects expression of endoplasmic reticulum-localized enzymes but is without effect on the mitochondrial system. At first sight, these findings seem to indicate that EE rather selectively suppresses the contribution of the neutral pathway of BS synthesis. In the intact rats only, this is apparently compensated for by increased flux via the acidic pathway. In spite of the unchanged BS synthesis rate in intact rats, however, BS pool size was significantly reduced, as previously reported by Davis and Kern (4). This may indicate that EE-treated rats are unable to upregulate hepatic BS synthesis adequately to compensate for fecal BS loss: it may be that EE treatment down-regulates the recently identified intestinal Na^+-dependent BS transporter (ibst) similar to its reported effects on expression of the hepatic Na^+-dependent BS transporter (Ntcp) (3, 8).

Our data also indicate that, in particular in EE-treated bile-diverted animals, steps prior to or beyond the initial hydroxylations may become rate-limiting, leading to a reduced BS synthesis in a situation when both CYP7A and CYP27 are upregulated in comparison with the control situation. This suggestion is supported by the absence of significant changes in biliary BS composition under these conditions.

A factor that may become rate-limiting in bile diverted animals is the supply of substrate, i.e. of (newly synthesized) cholesterol. We (26) and others (24) have shown that the contribution of *the novo* synthesized cholesterol to BS synthesis amounts up to 12 % in the intact rat with low BS synthesis and up to 40 - 50% in bile diverted rats. This shift in relative contribution of newly synthesized cholesterol to BS synthesis is most likely due to the fact that both synthetic processes are physically separated under normal conditions, i.e., are localized in different hepatocyte populations (25). This zonal distribution is largely lost when BS synthesis and cholesterol synthesis are derepressed by interruption of the enterohepatic circulation (25). As EE inhibits hepatic cholesterol synthesis (20-22), as confirmed in our study by decreased levels of *HMGS* mRNA in livers of EE-treated rats, it is conceivable that reduced substrate availability may contribute to reduced BS synthesis.

Another interesting finding of this study concerns the effects of EE on pool composition. A decreased contribution of cholate to the pool was found, as also previously reported by Kern *et al.* (5). Yet, synthesis of cholate was still appreciable in EE-treated intact rats. This is apparently in contrast to a previous study from our laboratory (10), in which we showed that the conversion of LDL-cholesterol to cholate is completely blocked in EE-treated rats. The combination of data implies that LDL-cholesterol is processed differently than cholesterol from other sources after EE administration. This may be a result of selective induction of LDL-receptors by EE in cell populations different from those expressing *CYP7A*, i.e. the periportal hepatocytes. Alternatively, it may be that in EE-treated rats LDL-cholesterol is preferably delivered to mitochondria for 27-hydroxylation rather than to the endoplasmic reticulum, where CYP7A resides.

The contribution of Δ^{22} β-muricholate to the pool was significantly increased in EE-treated rats. This newly identified rodent BS species is thought to represent a product of partial peroxisomal β-oxidation of the β-muricholate side chain (18,19,46). It is likely that the increased amounts of 6-hydroxylated BS species tentatively identified in bile of EE-treated rats by Kern *et al.* (5) actually represent Δ^{22} β-muricholate. Its increased contribution to the pool is probably the result of increased β-muricholate formation via the alternative pathway induced by EE: the fact that Δ^{22} β-muricholate disappears from the bile after interruption of the enterohepatic circulation is most likely explained by the assumption that β-muricholate can only be metabolized to Δ^{22} β-muricholate during entrohepatic cycling, i.e. after uptake from

the intestine and transport back to the liver. In this scenario, therefore, Δ^{22} β-muricholate cannot be considered a primary BS but represents a tertiary species.

In conclusion, our studies show that EE treatment selectively suppresses the initial step in the neutral pathway of BS synthesis controlled by CYP7A in rats, at least in part at transcriptional level. EE does not affect CYP27 activity and mRNA levels, probably leading to preferential BS synthesis via the alternative route. Yet, EE-effects on substrate availability and on enzymes further down the synthetic cascade may determine the ratios between the various end products of both synthetic pathways that are secreted into bile

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Chapter 8

General discussion & summary

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An elevated concentration of LDL cholesterol is an independent risk factor for cardiovascular disease. Since conversion of cholesterol into bile acids together with secretion of cholesterol in the bile is quantitatively the major pathway for excretion of cholesterol from the body, modulation of the bile acid synthetic pathway provides a way to affect serum cholesterol concentrations. More insight into the regulation of bile acid synthesis and the major enzymes involved in this process e.g. cholesterol 7α-hydroxylase and sterol 27-hydroxylase may explain the effects of different modulators on serum cholesterol levels and reveal new strategies for developing new pharmaceuticals which can enhance bile acid synthesis. In the studies described in this thesis we investigated the regulation of bile acid synthesis and its consequences for hepatic lipid metabolism.

In chapter 2 we investigated the role of different lipoproteins on bile acid synthesis in cultured rat hepatocytes. Among the lipoproteins used in this study, ßVLDL and IDL, both apo E-containing cholesterol-rich lipoproteins, induced cholesterol 7α-hydroxylase at the level of gene transcription, whereas the expression of sterol 27-hydroxylase was not affected. This indicates that both enzymes diverge with respect to regulation by exogenous cholesterol. The extent of stimulation of cholesterol 7α-hydroxylase is associated with the apo E content of the lipoprotein particle, which is important in the uptake of lipoprotein cholesterol. In the 348 nucleotides of the 5'-proximal promoter of cholesterol 7α-hydroxylase gene, which harbors a composite sequence responsive to various physiological signals, we localized a responsive site for ßVLDL-cholesterol. In this study we found a direct effect of cholesterol on the hepatocyte, though, these results do not fully exclude an indirect mechanism as proposed by others, in which stimulation of cholesterol 7α-hydroxylase by dietary cholesterol is ascribed to malabsorption of bile acids in the intestine, resulting in a reduced potential for bile acid-induced feedback. The molecular mechanism of induction of cholesterol 7α-hydroxylase by cholesterol or a metabolite remains to be clarified. The recently identified nuclear hormone receptor LXR seems to be a good candidate in this process. The results here demonstrate that atherogenic lipoproteins are efficiently routed to the bile acid synthesizing pathway and that they are not only substrate for bile acid synthesis, but that cholesterol or a metabolite can also play a regulatory role in the expression of cholesterol 7α-hydroxylase. Although sterol 27-hydroxylase expression is not

increased by cholesterol, the possibility that the increase in substrate leads to a higher amount of bile acids formed via this pathway, can not be excluded. *In vitro* studies have shown that the sterol 27-hydroxylase pathway may play a role in reverse cholesterol transport. In different extra-hepatic cells sterol 27-hydroxylase is able to convert cholesterol into bile acid intermediates that are more easily secreted from these cells. It would be interesting to know whether *in vivo* in the arterial wall an increased amount of lipoprotein cholesterol is also efficiently converted to these bile acid intermediates, which are efficiently transported to the liver. In this way sterol 27-hydroxylase can play an important anti-atherogenic role.

In our study described in chapter 3 we showed that in cells, in which the intracellular cholesterol pool is further enhanced by the simultaneous addition of ßVLDL with the ACAT inhibitor avasimibe, cholesterol 7α-hydroxylase mRNA levels were even higher than with ßVLDL alone. In these cells that had an even higher amount of regulatory cholesterol than cells which were incubated with ßVLDL alone, sterol 27-hydroxylase expression was still not affected. Cholesterol balance experiments in rat hepatocytes showed that the inhibition of ACAT causes a change in the metabolic pathway of cholesterol by reducing lipid secretion and storage increasing the supply of free cholesterol as a substrate and inducer of cholesterol 7α-hydroxylase, resulting in the enhanced production of bile acids. We further investigated the effect of this novel ACAT inhibitor on bile acid synthesis *in vivo* in the rat. Here the situation is more complicated, since the effects on intestinal ACAT can also play a role. Avasimibe was shown to be a potent lipid-lowering drug in rats. Under some dietary circumstances, in which there is sufficient cholesterol supply, avasimibe was indeed able to increase cholesterol 7α-hydroxylase expression. We found that there was no increase in the ratio of biliary excreted cholesterol to bile acids, indicating that ACAT inhibition does not result in a more lithogenic bile. Proper disposal of cholesterol into the bile acid synthetic pathway may contribute to the potent lipid-lowering effects of avasimibe in the rat. However, the primary hypocholesterolemic effect of avasimibe is caused by its decreasing effect on cholesterol absorption by the inhibition of intestinal ACAT, thereby reducing the transport of cholesterol to the liver.

Unfiltered coffee brews, which contain cafestol and kahweol, have been reported to markedly increase serum cholesterol levels in humans. We investigated the biochemical background of the cholesterol-raising effect of cafestol. In cultured rat hepatocytes (Chapter 4) cafestol suppressed bile acid synthesis by the down-

regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene expression and by direct inhibition of cholesterol 7α-hydroxylase enzyme activity. Suppression of bile acid synthesis resulted in a decreased expression of the hepatic LDL-receptor, probably due to an increase in the hepatic regulatory pool of cholesterol. This study provided evidence that the primary effect of cafestol is mediated on lipid metabolism in the hepatocyte. These effects were validated *in vivo* (Chapter 5). So far, no suitable animal model has been found for studying the mechanism of action of cafestol, since various routinely used animal models did not respond to this coffee diterpene as humans do. We showed for the first time that in mice cafestol caused an increase in serum cholesterol similar to that observed in humans, making this species a good model for investigating the biochemical background of the cholesterol-raising effect of cafestol in humans. Cafestol increased serum cholesterol levels in apoE*3-Leiden, heterozygous LDL-receptor deficient, and in wild-type mice, mainly in the VLDL and IDL fraction. The mechanism of action of cafestol was further studied in apoE*3-Leiden transgenic mice. In these mice, cafestol decreased bile acid synthesis, reflected by a reduction in the total amount of fecal bile acids excreted, by down-regulation of expression of enzymes involved in the neutral as well as in the alternative bile acid synthetic pathway. The consequent increase of hepatic cholesterol resulted in a decline in LDL-receptor mRNA levels and was removed from the liver by an increased secretion of VLDL cholesteryl esters. This may provide an explanation for the cholesterol-raising effect of unfiltered coffee in humans.

Two different modes of inhibition of bile acid synthesis were found in our study. On the one hand, cafestol suppressed bile acid synthesis by a direct inhibitory effect on cholesterol 7α-hydroxylase, which is probably based on its structural similarity with oxysterols, which have also been reported to be direct inhibitors of cholesterol 7α-hydroxylase enzyme activity. On the other hand, cafestol resembles sterols and it is therefore conceivable that inhibition of gene transcription by cafestol is regulated via, as yet unidentified, sequences within the cholesterol 7α-hydroxylase and sterol 27-hydroxylase promoter. Additional experiments, including functional analysis of the regulatory regions of the genes encoding cholesterol 7α-hydroxylase and sterol 27-hydroxylase will be necessary to elucidate the precise molecular mechanism of transcriptional repression of these genes by cafestol. In addition, in this thesis we studied the effects of cafestol on cholesterol metabolism, but it is also interesting to know how cafestol influences triglyceride metabolism, since our study

clearly demonstrated that the amount of liver triglycerides in the cafestol-treated group was remarkably reduced, suggesting an effect of cafestol on hepatic triglyceride metabolism in addition to its effect on cholesterol metabolism.

On the basis of our data, the next step would be to study the effects of cafestol on bile acid synthesis in humans by i.e. stable isotope experiments or analysis of fecal bile acid excretion. We found only a slight decrease in the amount of neutral sterols secreted, resulting in a change in the ratio between bile acids and cholesterol in bile, consequently leading to an increased lithogenicity index. A possible adverse effect of cafestol could be the formation of cholesterol gallstones, since an increased lithogenicity index increases the incidence of cholesterol gallstones. Most epidemiological studies did not show a correlation between coffee consumption and an increased prevalence of gallstone formation. The absence of such a correlation could be due to the method of coffee preparation. Nowadays, drinking unfiltered coffee e.g. espresso, Turkish coffee, French press coffee and cafetière coffee is becoming increasingly popular. It is advisible, therefore, to limit the intake of unfiltered coffee in patients with elevated cholesterol levels.

Down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase was also found by fibrates (Chapter 6). Fibrates are drugs that are widely used in the treatment of hyperlipidemia. These drugs lower triglyceride levels and increase HDL cholesterol in hyperlipidemic patients. However, side effects are also reported, i.e. long-term treatment with fibrates decreases the excretion of bile acids causing adverse changes in the biliary lipid composition and leading to an increased incidence of cholesterol gallstones. We investigated the mechanism of action of fibrates on bile acid synthesis and demonstrated that fibrates decrease bile acid synthesis by down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase expression. The decline in bile acid synthesis is mediated via activation of PPARα resulting in a decreased transcription of genes involved in bile acid synthesis. PPARα is also somehow involved in maintaining basal cholesterol 7α-hydroxylase expression, since knocking out PPARα resulted in a 3-fold diminution of basal cholesterol 7α-hydroxylase mRNA levels. An explanation may be that PPARα is involved in the sequestration of a (co)repressor that negatively regulates cholesterol 7α-hydroxylase gene transcription and/or that PPARα may interfere with other nuclear transcription factors mediating their effect by binding to the same responsive site as PPARα. Further studies are necessary to elucidate this phenomenon. Site-directed mutagenesis revealed that the DR-1(-146/-134), located

in the proximal promoter of cholesterol 7α-hydroxylase, is the target sequence for PPARα. This sequence is closely situated to/or overlaps with other responsive sites for various physiological signals i.e. bile acids, hormones and HNF-4. Therefore, PPARα may interfere with other nuclear transcription factors mediating their effect by binding to this responsive site. We showed that PPARα interferes with HNF-4 mediated activation of cholesterol 7α-hydroxylase gene transcription. A possibility for suppression of PPARα-mediated transcription of cholesterol 7α-hydroxylase could be via competition with HNF-4 for the DNA binding site or down-regulation of HNF-4 expression, as has been shown for fibrate action in other negatively-regulated genes. Otherwise, negative regulation of the expression of cholesterol 7α-hydroxylase could be exerted through the interaction of PPARα with a so-called negative PPRE. Additional experiments, including further functional analysis of the regulatory regions of the gene encoding cholesterol 7α-hydroxylase will be necessary to elucidate the precise mechanism of transcriptional repression of cholesterol 7α-hydroxylase gene expression by PPARα.

So, fibrates facilitate lower plasma lipid levels but paradoxically decrease bile acid synthesis and increase the secretion of cholesterol leading to gallstone formation. In patients who are more susceptible to cholesterol gallstone formation it is important to take notice of this disadvantage of these lipid-lowering drugs. Combination therapy of fibrates with compounds which can increase bile acid synthesis e.g. bile acid-binding resins is a possible treatment for these patients to reduce the risk of cholesterol gallstone formation.

In the last study described in this thesis (Chapter 7) the effects of the synthetic sex steroid 17α-ethinylestradiol in rats, which is a good model for estrogen-induced cholestasis, on bile acid synthesis were further investigated. Administration of ethinylestradiol to rats with an intact enterohepatic circulation resulted in a decrease in bile acid pool size and a change in the composition of the pool without a change in bile acid synthesis. In contrast, in long-term bile-diverted rats, there is a clear reduction in bile acid synthesis due to ethinylestradiol treatment. In both intact and bile-diverted rats, ethinylestradiol decreased cholesterol 7α-hydroxylase enzyme activity, whereas mRNA levels were reduced only in bile-diverted animals probably due to the short treatment period. In contrast, sterol 27-hydroxylase was not affected in either condition. In the intact rat only, the suppression of the neutral pathway to bile acid synthesis is apparently compensated for by an increased flux of cholesterol via the acidic pathway, resulting in a change in the bile acid pool composition. In the

bile-diverted rats, it became clear that cholesterol synthesis and other enzymes involved in bile acid synthesis i.e. sterol 12α-hydroxylase and lithocholic acid 6β-hydroxylase were also affected by ethinylestradiol. Therefore, the effects of ethinylestradiol on substrate availability and on enzymes further down-stream in the synthetic cascade may also contribute to determining the ratios between the various end products of both bile acid synthetic pathways. This study underlines the importance of combining data on gene expression and enzyme activities with those on the actual metabolic fluxes.

Nederlandse samenvatting

Hart-en vaatziekten zijn de meest voorkomende aandoeningen in de westerse samenleving. Het merendeel van de patiënten met hart-en vaatziekten heeft arteriosclerose. Arteriosclerose wil zeggen verdikking en verharding van de slagaderwand als gevolg van stapeling van cholesterol, verbindweefseling en proliferatie van cellen. Hierdoor treedt vernauwing op van de slagader wat de toevoer van bloed naar de weefsels via de slagader bemoeilijkt. Dit kan ervoor zorgen dat er minder zuurstof in deze weefsels komt, waarna ze kunnen afsterven. Als dat in het hart gebeurt, spreken we over een hartinfarct en in de hersenen over een herseninfarct of beroerte. Er zijn verschillende oorzaken voor het ontstaan van arteriosclerose. Naast verhoogde bloeddruk en roken, is ook een hoge concentratie van cholesterol en triglyceriden in het bloed een belangrijke factor. Cholesterol en triglyceriden zijn echter ook essentieel voor ons lichaam. Triglyceriden bestaan uit vetzuren gebonden aan glycerol en zorgen voor de energievoorziening in de spieren en voor opslag van energie in het vetweefsel. Cholesterol dient als bouwsteen voor celmembranen en is het uitgangsproduct voor de productie van hormonen en galzuren.

Cholesterol en triglyceriden zijn moeilijk oplosbaar in het bloed en worden daarom in ons lichaam verpakt in zogenaamde lipoproteïnen, die zorgen voor het transport van deze componenten. De lipoproteïnen worden omgeven door een "mantel" van eiwitten (apolipoproteïnen), die ervoor zorgen, dat de vetten naar de juiste plaats van bestemming worden gebracht. Er bestaan verschillende lipoproteïnen die variëren in dichtheid. Dit is het gevolg van verschillen in de verhouding van de eiwitten ten opzichte van de vetten. In het darmweefsel wordt het vet, dat we via onze voeding binnen krijgen, verpakt in lipoproteïnen genaamd chylomicronen. Tijdens het transport door het lichaam worden er vetten aan de weefsels afgegeven na inwerking van enzymen (lipasen). Hierdoor worden de chylomicronen steeds kleiner. Datgene wat overblijft van dit deeltje wordt uiteindelijk afgevoerd naar de lever. De lever speelt in het vetmetabolisme een centrale rol. Het kan zelf op zijn beurt ook weer lipoproteïnen in de bloedbaan brengen. De lipoproteïnen, die door de lever worden gemaakt, zijn de zogenaamde "very-low-density-lipoproteïnen" (VLDL), die veel triglyceriden bevatten. Net als bij de chylomicronen zal door inwerking van lipasen het VLDL-deeltje steeds minder triglyceriden bevatten en zal hierdoor de dichtheid van het deeltje veranderen. Zo

gaat het VLDL via "intermediate-density-lipoprotein" (IDL) over in "low-density-lipoprotein" (LDL). Dit laatste deeltje is erg cholesterolrijk en een verhoogde concentratie van LDL in het bloed is een risico factor voor hart-en vaatziekten. LDL-deeltjes kunnen uit de bloedbaan gehaald worden, nadat ze "opgevangen" zijn door receptoren aan de oppervlakte van cellen. De hoeveelheid receptoren op de cellen wordt bepaald door de hoeveelheid al aanwezige cholesterol in de cel of door erfelijke factoren. Het LDL wordt voor het grootste gedeelte uit de bloedbaan gehaald door LDL-receptoren op de lever. Daarnaast gaat er ook een gedeelte naar andere weefsels waar de cholesterol o.a. kan dienen voor de productie van hormonen. Wanneer er te weinig receptoren zijn kan de concentratie van het LDL cholesterol in het bloed te hoog worden en kan er cholesterol in de vaatwand terecht komen. Hier kan het LDL gemodificeerd worden door oxidatie. Het gemodificeerde deeltje kan vervolgens opgenomen worden door de "opruimcellen", ook wel macrofagen genoemd. Deze ontwikkelen zich tot zogenaamde schuimcellen, en dit is de eerste stap in het ontstaan van arteriosclerose. Naast het "slechte" LDL-deeltje kunnen we ook spreken van het "goede" "high-density-lipoprotein" (HDL). HDL ontstaat bij de afbraak van chylomicronen en wordt ook door de lever gemaakt. HDL heeft het vermogen om cholesterol uit de vaatwand op te nemen en het vervolgens naar de lever te transporteren. Naast de toevoer van cholesterol uit de bloedbaan is de lever ook zelf in staat om cholesterol te maken. Dit gebeurt door een cascade van enzymen. Een belangrijk enzym in dit proces is HMG-CoA reductase.

De lever speelt een belangrijke rol in het verwijderen van cholesterol uit het lichaam. Na opname van cholesterol vanuit de bloedbaan kan de lever het op verschillende manieren verwerken. 1) Het kan in de lever opgeslagen worden, nadat het cholesterol eerst is omgezet door een enzym (ACAT genaamd) tot cholesterol esters. 2) Het kan ook de lever weer verlaten, doordat het ingepakt wordt in het VLDL-deeltje. 3) De belangrijkste route voor verwijdering van cholesterol uit het lichaam is door uitscheiding in de gal. Dit kan direct of na omzetting van cholesterol in galzuren, een proces dat ook plaatsvindt in de lever. Elke dag verliezen wij een gedeelte van de gal via de ontlasting. Op deze manier raken wij dus ook een gedeelte van de galzuren en cholesterol kwijt. Een groot gedeelte van de galzuren wordt weer opgenomen via de darm en teruggevoerd naar de lever. Er zijn twee routes belangrijk in de vorming van galzuren. De belangrijkste route, die uit een cascade van enzymatische reacties bestaat, begint met de omzetting van cholesterol in 7α-hydroxycholesterol door het enzym cholesterol 7α-hydroxylase. Dit is het snelheidsbepalende enzym in deze route die bij de mens leidt tot de vorming van de

galzuren cholaat en chenodeoxycholaat. De alternatieve route begint met de omzetting van cholesterol in 27-hydroxycholesterol door sterol 27-hydroxylase, dat voornamelijk leidt tot de vorming van het galzuur chenodeoxycholaat.

Stimulatie van de galzuursynthese kan gebruikt worden in een cholesterol-verlagende therapie. Dit leidt tot vermindering van hart-en vaatziekten. Om dit te bewerkstelligen wordt de terugvoer van galzuren naar de lever verminderd en zo de afvoer via de faeces vergroot. Er zijn al medicijnen (bijv. cholestyramine) op de markt die de galzuren in de darm kunnen binden, waarna tezamen met de galzuren worden uitgescheiden. Een nadeel van deze medicijnen is dat grote hoeveelheden moeten worden ingenomen. Daarnaast zijn er nogal wat bijwerkingen bekend van deze stoffen. Er wordt nu dan ook veel onderzoek verricht aan het verbeteren van deze medicijnen (zoals beschreven in hoofdstuk 1). Behalve de genoemde mogelijkheid om de afvoer te vergroten, zou een andere optie zijn het vergroten van de galzuursynthese in de lever. Meer inzicht in de regulatie van de galzuursynthese en de belangrijkste enzymen die hierbij betrokken zijn, cholesterol 7α-hydroxylase en sterol 27-hydroxylase, is daarvoor van belang. Ook wanneer stoffen de galzuursynthese remmen, zal dat consequenties hebben voor het cholesterolgehalte in het bloed. In de studies beschreven in dit proefschrift hebben we onderzoek gedaan naar de regulatie en beïnvloeding van de galzuursynthese en de consequentie daarvan op de cholesterol en vetstofwisseling.

Cholesterol uit de lipoproteïnen kan de aanmaak van galzuren stimuleren. Hoe dit precies gebeurt en of elk deeltje een zelfde mate van stimulatie laat zien hebben we onderzocht en beschreven in hoofdstuk 2. Het onderzoek werd uitgevoerd met gekweekte levercellen (hepatocyten) die geïsoleerd zijn uit de rat. De hepatocyten kunnen in kweek galzuren produceren, en bieden een goede mogelijkheid om te kijken wat er nu werkelijk in de cellen gebeurt. Aan de kweekschaaltjes werden verschillende lipoproteïnen toegevoegd, namelijk ßVLDL (dit is een erg cholesterol-rijk VLDL), IDL en HDL. De cholesterol-rijke lipoproteïnen ßVLDL en IDL zorgden voor een verhoging van de galzuursynthese, terwijl HDL geen effect had. Deze stimulatie was het gevolg van een verhoogde expressie van de cholesterol 7α-hydroxylase gen. Dit gen, gelegen in het DNA, kan de aanzet geven voor de aanmaak van een cholesterol 7α-hydroxylase eiwit. De regulatie van de voor eiwitten coderende genen blijkt over het algemeen plaats te vinden op het niveau van de transcriptie (het overschrijven van het DNA naar mRNA). In onze studie zagen we, dat zowel het mRNA als de enzymactiviteit van cholesterol 7α-hydroxylase verhoogd

waren, terwijl er geen effecten waren op het mRNA en enzymactiviteit van sterol 27-hydroxylase. Dit wil zeggen dat beide enzymen verschillen in de regulatie door cholesterol afkomstig uit cholesterol-rijke lipoproteïnen. Niet alle lipoproteïnen lieten een zelfde mate van stimulatie van cholesterol 7α-hydroxylase zien. De stimulatie was afhankelijk van de hoeveelheid apolipoproteïne E (apo E) op het lipoproteïne deeltje. Apo E speelt een belangrijke rol bij de opname van lipoproteïnen door de receptoren op de lever. Hoe meer apo E er op het deeltje zit, hoe beter het door de lever wordt opgenomen (geklaard), wat resulteert in een verhoogde genexpressie van cholesterol 7α-hydroxylase. In de flankerende gebieden van de genen liggen DNA sequenties (promoter), die door binding van bepaalde transcriptiefactoren de transcriptie van het gen kunnen reguleren. In het promotergebied van het cholesterol 7α-hydroxylase gen hebben we een sequentie gevonden die zorgt voor de regulatie van cholesterol 7α-hydroxylase door ßVLDL. Of cholesterol nu zelf een regulerende rol heeft of dat vervolgproducten die uit cholesterol gevormd worden hierbij een rol spelen, moet nog verder worden uitgezocht.

In hoofdstuk 3 hebben we de hoeveelheid cholesterol, dat beschikbaar is als substraat of als regulerende factor voor de galzuursynthese, nog verder verhoogd door de cellen te incuberen met ßVLDL in aanwezigheid van een nieuwe ACAT-remmer: avasimibe (ook wel CI-1011 genoemd). De ACAT-remmer zorgt ervoor dat omzetting van cholesterol in cholesterolesters geremd wordt. Hierdoor is er meer cholesterol beschikbaar voor de regulatie van de galzuursynthese. In de cellen waarin de hoeveelheid regulerend cholesterol nog sterker verhoogd was dan bij de incubaties met alleen ßVLDL zagen we nog steeds geen effecten op sterol 27-hydroxylase, terwijl de enzymactiviteit en mRNA niveaus van cholesterol 7α-hydroxylase juist nog sterker verhoogd waren. In de gekweekte hepatocyten zorgde avasimibe ervoor dat er een verschuiving optrad in de cholesterolbalans. Remming van ACAT zorgde voor een verminderde opslag en uitscheiding van cholesterolesters, terwijl de omzetting van cholesterol in galzuren toenam. Om een uitgebreider beeld te krijgen van wat avasimibe doet in het hele organisme (*in vivo*), hebben we studies gedaan, waarin ratten op verschillende diëten behandeld werden met avasimibe. *In vivo* is de situatie iets gecompliceerder, omdat avasimibe ook effecten heeft op ACAT in de darm en zo de opname van cholesterol kan remmen. Behandeling van de ratten met avasimibe liet op alle diëten een sterke daling zien van het cholesterol en triglyceride gehalte in het bloed en in de lever. Onder bepaalde voedingsomstandigheden, waarbij er voldoende cholesterol aanvoer was,

werd ook hier cholesterol 7α-hydroxylase gestimuleerd. Een belangrijke bevinding was ook dat de verzadiging van de gal door cholesterol, wat aanleiding kan geven tot galsteenvorming, niet was verhoogd. Uit deze studie kunnen we concluderen dat een "nette" afvoer van cholesterol door stimulatie van de galzuursynthese door avasimibe kan bijdragen aan het cholesterol verlagende effect van dit toekomstige medicijn. Maar het primaire effect van avasimibe bij de rat wordt veroorzaakt door een verminderde absorptie van cholesterol door remming van ACAT in de darm wat leidt tot een verminderd transport van cholesterol naar de lever.

Een ander onderdeel van de studies betrof onderzoek naar de biochemische achtergrond van de werking van stoffen op de cholesterolhuishouding en vooral het effect op de galzuursynthese.

Ongefilterde koffie, zoals bijv. Scandinavische gekookte koffie, cafetière-, Turkse en espresso koffie kunnen het cholesterolgehalte bij de mens in het bloed verhogen. Ongefilterde koffie bevat de koffiecomponenten cafestol en kahweol. Wanneer je een koffiefilter gebruikt, blijven cafestol en kahweol hierin hangen. Hoe het komt dat ongefilterde koffie het cholesterolgehalte verhoogt, hebben wij bestudeerd en beschreven in hoofdstuk 4 en 5. In gekweekte rattehepatocyten onderdrukte cafestol de galzuursynthese door de verminderde transcriptie van cholesterol 7α-hydroxylase en sterol 27-hydroxylase en door een directe remming van de cholesterol 7α-hydroxylase enzymactiviteit. De verminderde galzuursynthese leidde tot een verminderde expressie van de LDL-receptor, waarschijnlijk als gevolg van een stijgende hoeveelheid cholesterol in de cel. Daarnaast hebben we onderzocht of deze effecten ook *in vivo* plaatsvinden. Tot nu toe was er nog geen geschikt diermodel beschikbaar die een zelfde plasma cholesterol verhoging door cafestol liet zien zoals bij de mens. Wij hebben voor de eerste keer laten zien dat muizen op een vergelijkbare manier reageren op cafestol zoals mensen. In onze studie hebben we naast gewone muizen gebruik gemaakt van genetisch gemodificeerde muizen, nl de heterozygote LDL-receptor deficiënte muizen en de apoE*3-Leiden muizen. Deze genetisch gemodificeerde muizen hebben een lipoproteïnenprofiel dat vergelijkbaar is met dat van de mens. Cafestol verhoogde het serum cholesterolgehalte in alle drie de muizenstammen. In de apoE*3-Leiden muizen hebben we het mechanisme van het cholesterol-verhogende effect van cafestol verder onderzocht. Ook hier zagen we een afname in de galzuursynthese door verlaging van de expressie van enzymen betrokken in zowel de primaire als in de alternatieve route in de vorming van galzuren. Dit resulteerde in een verminderde hoeveelheid galzuren in de faeces van deze muizen. De afname in galzuurproductie

leidde tot een verhoogde uitscheiding van cholesterolesters in VLDL en tot een verminderde expressie van de LDL-receptor. Dus enerzijds zorgt een verminderde galzuursynthese ervoor dat er minder cholesterol door de lever wordt opgenomen en anderzijds zal het leiden tot een verhoogde afvoer van cholesterol in VLDL-deeltjes. Dit kan een verklaring zijn voor de verhoging van het serum cholesterolgehalte zoals dat gevonden wordt bij de mensen.

Fibraten zijn medicijnen die veel worden voorgeschreven aan mensen met hyperlipidemie. Deze medicijnen verlagen het triglyceriden gehalte en verhogen het HDL cholesterol. Naast alle gunstige effecten van deze medicijnen zijn er ook bijwerkingen bekend. Wanneer mensen voor een lange periode met fibraten behandeld worden, resulteert dit in een verminderde uitscheiding van galzuren en een verhoogde uitscheiding van cholesterol in de gal. Doordat de verhouding cholesterol t.o.v. galzuren in de gal verandert, is de gal oververzadigd met cholesterol wat leidt tot de vorming van galstenen. In onze studie hebben we gekeken naar het mechanisme dat ervoor zorgt dat de galzuursynthese omlaag gaat. De verlaging van de galzuursynthese was het gevolg van een afname in de transcriptie van cholesterol 7α-hydroxylase en sterol 27-hydroxylase. Fibraten kunnen de transcriptiefactor PPARα stimuleren en zo de transcriptie van verschillende genen beïnvloeden. Wij hebben onderzocht of de afname in de transcripitie van de genen betrokken bij de galzuurproductie ook door PPARα wordt gereguleerd. Daarvoor hebben we gebruik gemaakt van gewone (wildtype) muizen en muizen die het gen voor PPARα missen (de zogenaamde PPARα deficiënte muizen). Behandeling met ciprofibraat in de wildtype muizen zorgde voor een afname van cholesterol 7α-hydroxylase en sterol 27-hydroxylase expressie, terwijl in de PPARα deficiënte muizen dit effect volledig afwezig was. Dit is een aanwijzing voor de betrokkenheid van PPARα in de regulatie van de galzuursynthese door fibraten. Daarnaast hebben we door mutatie-analyse in het promotergebied van het cholesterol 7α-hydroxylase gen een sequentie gevonden die betrokken is bij de regulatie van het gen door PPARα. Wij concluderen dat een verminderde productie van galzuren onder invloed van fibraten kan bijdragen aan het toegenomen risico op galsteenvorming bij patiënten die met fibraten worden behandeld.

In de laatste studie beschreven in dit proefschrift hebben we de effecten van het vrouwelijke geslachtshormoon oestrogeen op de galzuursynthese bestudeerd. Ratten lieten na behandeling met het synthetische 17α-ethinyl estradiol een verandering in de uitscheiding van galzuren zien. De primaire route werd door 17α-

ethinyl estradiol geremd als gevolg van een afname in de enzymactiviteit en mRNA van cholesterol 7α-hydroxylase. Er waren geen effecten op sterol 27-hydroxylase waar te nemen. Deze verandering leidde bij intacte ratten echter niet tot een verminderde galzuursynthese, waarschijnlijk veroorzaakt doordat een groot deel wordt overgenomen door de alternatieve route. Dit vormt ook een verklaring voor de verandering in galzuurcompositie in deze dieren. De verandering in ratios tussen de verschillende galzuren zou ook verklaard kunnen worden doordat enzymen verderop in de route beïnvloed zijn door 17α-ethinyl estradiol.

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Abbreviations

ACAT	acyl-coenzyme A:cholesterol-acyltransferase
Apo	apolipoprotein
BARE	bile acid response element
BARP	bile acid responsive protein
BTE	basic transcription element
BZIP	basic leucine zipper protein
C	cholic acid
CAT	chloramphenicol acetyltransferase
CBD	complete biliary diverted
CDC	chenodeoxycholic acid
CE	cholesteryl ester
C/EBP	CCAAT-enhancer binding protein
CEH	cholesteryl ester hydrolase
CHD	coronary heart disease
CTX	cerebrotendinous xanthomatosis
DBP	albumin promoter D-site binding protein
DC	deoxycholic acid
DMSO	dimethylsulfoxide
DR	direct repeat
DTT	dithiothreitol
EE	17α-ethinyl estradiol
EHC	enterohepatic circulation
FH	familial hypercholesterolemia
GAPDH	glyceraldehyde-3-phosphate
GRE	glucocorticoid responsive element
HDL	high-density-lipoprotein
HDC	hyodeoxycholic acid
HFC/0	high fat /high cholesterol
HFC/0.5	high fat/high cholesterol with 0.5% cholate
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI_x	hydrophobicity index
HLF	human leukemia factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
HNF	hepatic nuclear factor

HRE	hormone responsive element
IDL	intermediate-density-lipoprotein
LC	litocholic acid
LDL	low-density-lipoprotein
LFC	low fat/low cholesterol
LPDS	lipoprotein deficient serum
LXR	liver X receptor
MC	muricholic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue)
PBS	phosphate buffered saline
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
RAR	retinoic acid receptor
RXR	retinoid X receptor
SCP	sterol carrier protein
SDS-PAGE	SDS-polyacrylamide-gel-electrophoresis
SREBP	sterol regulatory element binding protein
StAR	steroidogenic acute regulatory protein
TG	triglyceride
TRE	thyroid regulatory element
UDC	ursodeoxycholic acid
UTR	untranslated region
VLDL	very-low-density lipoprotein
ßVLDL	ß-migrating very-low-density lipoprotein

Curriculum Vitae

Sabine Maria Post werd geboren op 17 februari 1971 in Bussum. In 1989 behaalde zij haar VWO diploma aan het Sint Vituscollege te Bussum. In augustus van datzelfde jaar werd begonnen aan de studie Biologie aan de Universiteit van Utrecht. De propedeuse werd in augustus van 1990 behaald, waarna vervolgens het doctoraal examen in januari 1995 werd afgelegd. In het kader van het doctoraal examen werden twee onderzoeksstages gedaan waarvan één op de afdeling Electronenmicroscopie en Structuuranalyse van de vakgroep Moleculaire Celbiologie aan de Universiteit van Utrecht (Dr. P.J. Rijken en Prof. Dr. A.J. Verkleij). De tweede stage werd verricht bij de vakgroep Infectieziekten & Immunologie afdeling Virologie aan de Universiteit Utrecht (Dr. A.A.F. de Vries en Prof. Dr. Rottier).
Van mei 1995 tot mei 1999 was zij aangesteld als assistent in opleiding aan het Leids Universitair Medisch Centrum op de afdeling Inwendige Geneeskunde op een door de Nederlandse Hartstichting gesubsidieerd project (#94.049). Tijdens deze periode was zij gedetacheerd bij TNO Preventie en Gezondheid en werkzaam in het Gaubius Laboratorium te Leiden. Gedurende deze periode werd onderleiding van Dr. H.M.G. Princen het in dit proefschrift beschreven onderzoek verricht.
Vanaf 1 augustus 1999 is zij voor een periode van 4 jaar aangesteld als postdoc bij TNO Preventie en Gezondheid in het Gaubius Laboratorium te Leiden in de groep van Dr. H.M.G. Princen. In deze periode zal zij onderzoek verrichten, in het kader van een door de Nederlandse Hartstichting gesubsidieerd project (#97.116), aan de regulatie van de galzuursynthese.